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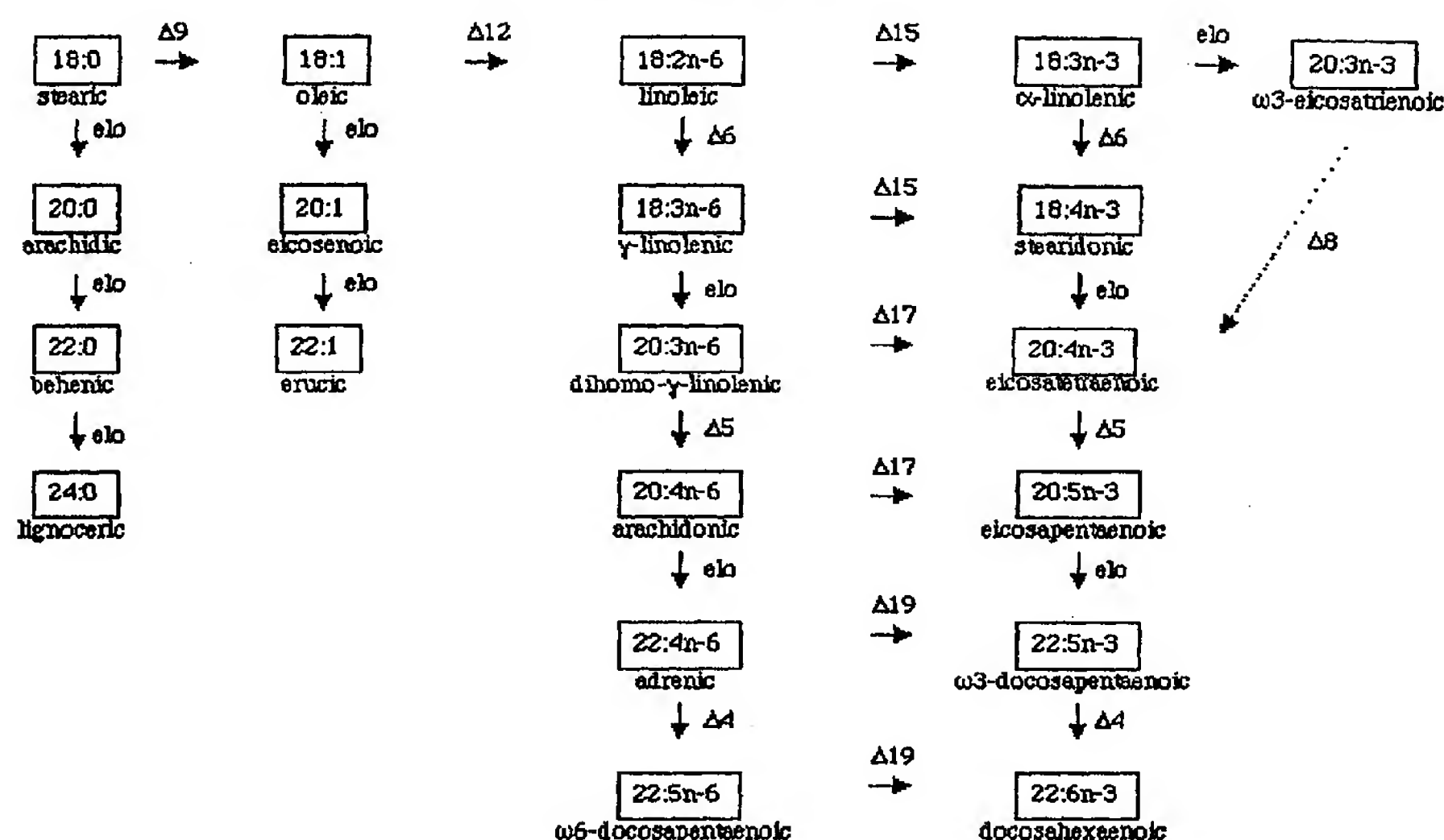
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(54) Title: DESATURASE GENES AND USES THEREOF

Fatty Acid Biosynthetic Pathway



(57) Abstract: The subject invention relates to the identification of genes involved in the desaturation of polyunsaturated fatty acids at carbon 5 (i.e., "Δ5-desaturase") and at carbon 6 (i.e., "Δ6-desaturase") and to uses thereof. In particular, Δ5-desaturase may be utilized, for example, in the conversion of dihomog-γ-linolenic acid (DGLA) to arachidonic acid (AA) and in the conversion of 20:4n-3 to eicosapentaenoic acid (EPA). Delta-6 desaturase may be used, for example, in the conversion of linoleic (LA) to γ-linolenic acid (GLA). AA or polyunsaturated fatty acids produced therefrom may be added to pharmaceutical compositions, nutritional compositions, animal feeds, as well as other products such as cosmetics.



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## DESATURASE GENES AND USES THEREOF

BACKGROUND OF THE INVENTIONTechnical Field

The subject invention relates to the identification and isolation of genes that encodes enzymes (i.e., Thraustochytrium aureum  $\Delta 5$ -desaturase, Saprolegnia diclina  $\Delta 5$ -desaturase, Saprolegnia diclina  $\Delta 6$ -desaturase and Isochrysis galbana  $\Delta 5$ -desaturase) involved in the synthesis of polyunsaturated fatty acids and to uses thereof. In particular,  $\Delta 5$ -desaturase catalyzes the conversion of, for example, dihomo- $\gamma$ -linolenic acid (DGLA) to arachidonic acid (AA) and (n-3)-eicosatetraenoic acid (20:4n-3) to eicosapentaenoic acid (20:5n-3). Delta-6 desaturase catalyzes the conversion of, for example,  $\alpha$ -linolenic acid (ALA) to stearidonic acid (STA). The converted products may then be utilized as substrates in the production of other polyunsaturated fatty acids (PUFAs). The product or other polyunsaturated fatty acids may be added to pharmaceutical compositions, nutritional composition, animal feeds as well as other products such as cosmetics.

Background Information

Desaturases are critical in the production of long-chain polyunsaturated fatty acids that have many important functions. For example, polyunsaturated fatty acids (PUFAs) are important components of the plasma membrane of a cell, where they are found in the form of phospholipids. They also serve as precursors to mammalian prostacyclins, eicosanoids, leukotrienes and prostaglandins. Additionally, PUFAs are necessary for the proper development of the developing infant brain as well as for tissue formation and repair. In view of the biological significance of PUFAs, attempts are being made

to produce them, as well as intermediates leading to their production, in an efficient manner.

A number of enzymes are involved in PUFA biosynthesis in addition to  $\Delta 5$ -desaturase and  $\Delta 6$ -desaturase. For example, elongase (elo) catalyzes the conversion of  $\gamma$ -linolenic acid (GLA) to dihomogamma-linolenic acid (DGLA) and of stearidonic acid (18:4n-3) to (n-3)-eicosatetraenoic acid (20:4n-3). Linoleic acid (LA, 18:2- $\Delta 9,12$  or 18:2n-6) is produced from oleic acid (18:1- $\Delta 9$ ) by a  $\Delta 12$ -desaturase. GLA (18:3- $\Delta 6,9,12$ ) is produced from linoleic acid by a  $\Delta 6$ -desaturase.

It must be noted that animals cannot desaturate beyond the  $\Delta 9$  position and therefore cannot convert oleic acid into linoleic acid. Likewise,  $\alpha$ -linolenic acid (ALA, 18:3- $\Delta 9,12,15$ ) cannot be synthesized by mammals. However,  $\alpha$ -linolenic acid can be converted to stearidonic acid (STA, 18:4- $\Delta 6,9,12,15$ ) by a  $\Delta 6$ -desaturase (see PCT publication WO 96/13591 and The FASEB Journal, Abstracts, Part I, Abstract 3093, page A532 (Experimental Biology 98, San Francisco, CA, April 18-22, 1998); see also U.S. Patent No. 5,552,306), followed by elongation to (n-3)-eicosatetraenoic acid (20:4- $\Delta 8,11,14,17$ ) in mammals and algae. This polyunsaturated fatty acid (i.e., 20:4- $\Delta 8,11,14,17$ ) can then be converted to eicosapentaenoic acid (EPA, 20:5- $\Delta 5,8,11,14,17$ ) by a  $\Delta 5$ -desaturase, such as that of the present invention. Other eukaryotes, including fungi and plants, have enzymes which desaturate at carbon 12 (see PCT publication WO 94/11516 and U.S. Patent No. 5,443,974) and carbon 15 (see PCT publication WO 93/11245). The major polyunsaturated fatty acids of animals therefore are either derived from diet and/or from desaturation and elongation of linoleic acid or  $\alpha$ -linolenic acid. In view of these difficulties, it is of significant interest to isolate genes involved in PUFA synthesis from species that naturally produce



these fatty acids and to express these genes in a microbial, plant, or animal system which can be altered to provide production of commercial quantities of one or more PUFAs.

One of the most important long chain PUFAs, noted above,  
5 is arachidonic acid (AA). AA is found in filamentous fungi and can also be purified from mammalian tissues including the liver and adrenal glands. As noted above, AA production from dihomog-  
linolenic acid is catalyzed by a  $\Delta 5$ -desaturase. EPA is  
another important long-chain PUFA. EPA is found in fungi and  
10 also in marine oils. As noted above, EPA is produced from (n-3)-eicosatetraenoic acid and is catalyzed by a  $\Delta 5$ -desaturase. In view of the above discussion, there is a definite need for the  $\Delta 5$ -desaturase and  $\Delta 6$ -desaturase enzymes, the respective genes encoding these enzymes, as well as recombinant methods of  
15 producing these enzymes. Additionally, a need exists for oils containing levels of PUFAs beyond those naturally present as well as those enriched in novel PUFAs. Such oils can only be made by isolation and expression of the  $\Delta 5$ -desaturase and  $\Delta 6$ -desaturase genes.

20 All U.S. patents, priority documents and publications referred to herein are hereby incorporated in their entirety by reference.

#### SUMMARY OF THE INVENTION

25 The present invention includes an isolated nucleotide sequence or fragment comprising or complementary to at least about 50% of a nucleotide sequence comprising SEQ ID NO:13 (Figure 2), SEQ ID NO:19 (Figure 4), SEQ ID NO:28 (Figure 6), SEQ ID NO:30 (Figure 8), SEQ ID NO:32 (Figure 10) or SEQ ID  
30 NO:34 (Figure 14). In particular, the isolated nucleotide sequence may be represented by SEQ ID NO:13, SEQ ID NO:19, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32 or SEQ ID NO:34. These

sequences may encode a functionally active desaturase which utilizes a polyunsaturated fatty acid as a substrate.

Furthermore, the present invention encompasses an isolated nucleotide sequence or fragment comprising or complementary to  
5 a nucleotide sequence encoding a polypeptide having desaturase activity and having at least 50% identity or similarity to an amino acid sequence selected from the group consisting of SEQ ID NO:20, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33 and SEQ ID NO:35.

10 The nucleotide sequences may be derived from, for example, a fungus such as Saprolegnia diclina (SEQ ID NO:13 and SEQ ID NO:19) or Thraustochytrium aureum (SEQ ID NO:28, SEQ ID NO:30 and SEQ ID NO:32), or an algae such as, for example, Isochrysis galbana (SEQ ID NO:34).

15 The present invention also includes purified proteins or polypeptides (SEQ ID NO:14 (Figure 3), SEQ ID NO:20 (Figure 5), SEQ ID NO:29 (Figure 7), SEQ ID NO: 31 (Figure 31), SEQ ID NO:33 (Figure 11) and SEQ ID NO:35 (Figure 15)) encoded by the nucleotide sequences referred to above.

20 Additionally, the present invention includes a purified polypeptide which desaturates polyunsaturated fatty acids at carbon 5 or carbon 6 and has at least about 50% amino acid identity or similarity to the amino acid sequence of the purified proteins referred to directly above (i.e., SEQ ID  
25 NO:14, SEQ ID NO:20, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33 and SEQ ID NO:35).

Furthermore, the present invention also encompasses a method of producing a desaturase (i.e.,  $\Delta 5$  or  $\Delta 6$ ). This method comprises the steps of: a) isolating the nucleotide sequence  
30 comprising SEQ ID NO:19, SEQ ID NO:28, SEQ ID NO:13, SEQ ID NO:30, SEQ ID NO:32, or SEQ ID NO:34 as appropriate; b) constructing a vector comprising: i) the isolated nucleotide sequence operably linked to ii) a promoter or some type of

regulatory sequence; and c) introducing the vector into a host cell for a time and under conditions sufficient for expression of the  $\Delta 5$ -desaturase or  $\Delta 6$ -desaturase, as appropriate. The host cell may be, for example, a eukaryotic cell or a  
5 prokaryotic cell. In particular, the prokaryotic cell may be, for example, E. coli, Cyanobacteria or B. subtilis. The eukaryotic cell may be, for example, a mammalian cell, an insect cell, a plant cell or a fungal cell (e.g., a yeast cell such as Saccharomyces cerevisiae, Saccharomyces carlsbergensis,  
10 Candida spp., Lipomyces starkey, Yarrowia lipolytica, Kluyveromyces spp., Hansenula spp., Trichoderma spp. or Pichia spp.).

Additionally, the present invention also encompasses a vector comprising: a) a nucleotide sequence as represented by  
15 SEQ ID NO:13, SEQ ID NO:19, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32 or SEQ ID NO:34 operably linked to b) a promoter or regulatory sequence. The invention also includes a host cell comprising this vector. The host cell may be, for example, a eukaryotic cell or a prokaryotic cell. Suitable eukaryotic  
20 cells and prokaryotic cells are as defined above.

Moreover, the present invention also includes a plant cell, plant or plant tissue comprising the above vector, wherein expression of the nucleotide sequence of the vector results in production of at least one polyunsaturated fatty  
25 acids by the plant cell, plant or plant tissue. The polyunsaturated fatty acid may be, for example, selected from the group consisting of AA, EPA, GLA or STA, depending upon whether the nucleotide sequence encodes a  $\Delta 5$ - or  $\Delta 6$ -desaturase. The invention also includes one or more plant oils or acids  
30 expressed by the above plant cell, plant or plant tissue.

Additionally, the present invention also encompasses a transgenic plant comprising the above vector, wherein expression of the nucleotide sequence of the vector results in

production of a polyunsaturated fatty acid in seeds of the transgenic plant.

Also, the invention includes a mammalian cell comprising the above vector wherein expression of the nucleotide sequence of the vector results in production of altered levels of AA, EPA, GLA and/or STA when the cell is grown in a culture media comprising a fatty acid selected from the group consisting of, for example, LA, ALA, DGLA and ETA.

It should also be noted that the present invention encompasses a transgenic, non-human mammal whose genome comprises a DNA sequence encoding a  $\Delta 5$ -desaturase or a  $\Delta 6$ -desaturase, operably linked to a promoter or regulatory sequence. The DNA sequence may be represented by SEQ ID NO:13 ( $\Delta 6$ ), SEQ ID NO:19 ( $\Delta 5$ ), SEQ ID NO:28 ( $\Delta 5$ ), SEQ ID NO:30 ( $\Delta 5$ ), SEQ ID NO:32 ( $\Delta 6$ ) and SEQ ID NO:34 ( $\Delta 5$ ). Additionally, the present invention includes a fluid (e.g., milk) produced by the transgenic, non-human mammal wherein the fluid comprises a detectable level of at least  $\Delta 5$ -desaturase or at least  $\Delta 6$ -desaturase, as appropriate.

Additionally, the present invention includes a method (i.e., "first" method) for producing a polyunsaturated fatty acid comprising the steps of: a) isolating the nucleotide sequence represented by, for example, SEQ ID NO:19, SEQ ID NO:28, SEQ ID NO:30 or SEQ ID NO:34; b) constructing a vector comprising the isolated nucleotide sequence; c) introducing the vector into a host cell under time and conditions sufficient for expression of  $\Delta 5$ -desaturase enzyme; and d) exposing the expressed human  $\Delta 5$ -desaturase enzyme to a substrate polyunsaturated fatty acid in order to convert the substrate to a product polyunsaturated fatty acid. The substrate polyunsaturated fatty acid may be, for example, DGLA or 20:4n-3 and the product polyunsaturated fatty acid may be, for example, AA or EPA, respectively. This method may further comprise the step of exposing the product polyunsaturated fatty acid to an

elongase or desaturase in order to convert the product polyunsaturated fatty acid to another polyunsaturated fatty acid (i.e., "second" method). In this method containing the additional step (i.e., "second" method), the product

5 polyunsaturated fatty acid may be, for example, AA or EPA, and the "another" polyunsaturated fatty acid may be adrenic acid or (n-3)-docosapentaenoic acid, respectively. The method containing the additional step may further comprise a step of exposing the another polyunsaturated fatty acid to an  
10 additional desaturase or elongase in order to convert the another polyunsaturated fatty acid to a final polyunsaturated fatty acid (i.e., "third" method). The final polyunsaturated fatty acid may be, for example, (n-6)-docosapentaenoic acid or docosahexaenoic (DHA) acid.

15 Additionally, the present invention includes a method for producing a polyunsaturated fatty acid comprising the steps of:

a) isolating the nucleotide sequence represented by SEQ ID NO:13 or SEQ ID NO:33; b) constructing a vector comprising the isolated nucleotide sequence; c) introducing the vector into a

20 host cell for a time and under conditions sufficient for expression of  $\Delta 6$ -desaturase enzyme; and d) exposing the expressed  $\Delta 6$ -desaturase enzyme to a substrate polyunsaturated fatty acid in order to convert the substrate to a product polyunsaturated fatty acid. The substrate polyunsaturated

25 fatty acid may be, for example, LA or ALA, and the product polyunsaturated fatty acid may be, for example, GLA or STA, respectively. This method may further comprise the step of exposing the product polyunsaturated fatty acid to an elongase (or desaturase) in order to convert the product polyunsaturated  
30 fatty acid to another polyunsaturated fatty acid. In this method containing the additional step, the product polyunsaturated fatty acid may be, for example, GLA or STA, and the "another" polyunsaturated fatty acid may be DGLA or eicosatetraenoic acid (ETA), respectively. The method



containing the additional step may further comprise a step of exposing the another polyunsaturated fatty acid to an additional desaturase (or elongase) in order to convert the another polyunsaturated fatty acid to a final polyunsaturated fatty acid. The final polyunsaturated fatty acid may be, for example, AA or EPA.

The present invention also encompasses a nutritional composition comprising at least one polyunsaturated fatty acid selected from the group consisting of the product polyunsaturated fatty acid produced according to the methods described above, the another polyunsaturated fatty acid produced according to the methods described above, and the final polyunsaturated fatty acid produced according to the methods described above. The product polyunsaturated fatty acid may be, for example, AA, EPA, GLA or STA, depending upon whether one is using a  $\Delta 5$ - or  $\Delta 6$ -desaturase nucleotide sequence. The another polyunsaturated fatty acid may be, for example, adrenic acid, (n-3)-docosapentaenoic acid, DGLA and EPA, again depending upon whether one is using a  $\Delta 5$ - or  $\Delta 6$ -desaturase nucleotide sequence. The final polyunsaturated fatty acid may be, for example, (n-6)-docosapentaenoic acid, DHA, AA or EPA, again, depending upon whether one is using a  $\Delta 5$ - or  $\Delta 6$ -desaturase nucleotide sequence.

The present invention also includes a pharmaceutical composition comprising 1) at least one polyunsaturated fatty acid (PUFA) selected from the group consisting of the "product" PUFA produced according to the methods described above, the "another" PUFA produced according to the methods described above, or the "final" PUFA produced according to the methods described above and 2) a pharmaceutically acceptable carrier.

Additionally, the present invention encompasses an animal feed or cosmetic comprising at least one PUFA selected from the group consisting of the product PUFA produced according to the methods described above, the another PUFA produced according to



the methods described above and the final PUFA produced according to the methods described above. These PUFAs have been listed above and are exemplified in Figure 1.

Additionally, the present invention encompasses a method  
5 of preventing or treating a condition caused by insufficient intake of polyunsaturated fatty acids comprising administering to the patient the nutritional composition above in an amount sufficient to effect prevention or treatment.

It should also be noted that each nucleotide and amino  
10 acid sequence referred to herein has been assigned a particular sequence identification number. The Sequence Listing (which is found herein) lists each such sequence and its corresponding number.

#### 15 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the fatty acid biosynthetic pathway and the roles of  $\Delta 5$ -desaturase and  $\Delta 6$ -desaturase in this pathway.

Figure 2 illustrates the nucleotide sequence encoding  $\Delta 6$ -  
20 desaturase of Saprolegnia diclina (ATCC 56851) (SEQ ID NO:13).

Figure 3 illustrates the amino acid sequence of  $\Delta 6$ -desaturase of Saprolegnia diclina (ATCC 56851) (SEQ ID NO:14).

Figure 4 illustrates the nucleotide sequence encoding  $\Delta 5$ -desaturase of Saprolegnia diclina (ATCC 56851) (SEQ ID NO:19).

25 Figure 5 illustrates the amino acid sequence of  $\Delta 5$ -desaturase of Saprolegnia diclina (ATCC 56851) (SEQ ID NO:20).

Figure 6 illustrates the nucleotide sequence encoding  $\Delta 5$ -desaturase of Thraustochytrium aureum (ATCC 34304) (SEQ ID NO:28).

30 Figure 7 illustrates the amino acid sequence of  $\Delta 5$ -desaturase of Thraustochytrium aureum (ATCC 34304) (SEQ ID NO:29).

Figure 8 illustrates the nucleotide sequence encoding  $\Delta 5$ -desaturase from Thraustochytrium aureum (BICC7091) (SEQ ID NO:30).

Figure 9 illustrates the translated amino acid sequence of  $\Delta 5$ -desaturase from Thraustochytrium aureum (BICC7091) (SEQ ID NO:31).

Figure 10 illustrates the nucleotide sequence encoding  $\Delta 6$ -desaturase from Thraustochytrium aureum (BICC7091) (SEQ ID NO:32).

Figure 11 illustrates the translated amino acid sequence of  $\Delta 6$ -desaturase from Thraustochytrium aureum (BICC7091) (SEQ ID NO:33).

Figure 12 illustrates the  $\Delta 5$ -desaturase amino acid sequence identity between pRAT-2a and pRAT-2c clones.

Figure 13 illustrates the  $\Delta 6$ -desaturase amino acid sequence identity between pRAT-1a and pRAT-1b clones.

Figure 14 illustrates the nucleotide sequence encoding  $\Delta 5$ -desaturase gene from Isochrysis galbana CCMP1323 (SEQ ID NO:34).

Figure 15 illustrates the translated amino acid sequence from  $\Delta 5$ -desaturase from Isochrysis galbana CCMP1323 (SEQ ID NO:35).

#### DETAILED DESCRIPTION OF THE INVENTION

The subject invention relates to the nucleotide and translated amino acid sequences of a  $\Delta 5$ -desaturase gene derived from Saprolegnia diclina, a  $\Delta 6$ -desaturase gene derived from Saprolegnia diclina, two  $\Delta 5$ -desaturase genes derived from Thraustochytrium aureum and a  $\Delta 6$ -desaturase gene derived from Isochrysis galbana. Furthermore, the subject invention also includes uses of these genes and of the enzymes encoded by these genes. For example, the genes and corresponding enzymes

may be used in the production of polyunsaturated fatty acids such as, for instance, arachidonic acid, eicosapentaenoic acid, and/or adrenic acid which may be added to pharmaceutical compositions, nutritional compositions and to other valuable products.

The  $\Delta$ 5-Desaturase Genes, The  $\Delta$ 6-Desaturase Genes, and Enzymes Encoded Thereby

As noted above, the enzymes encoded by the  $\Delta$ 5-desaturase genes and the  $\Delta$ 6-desaturase genes of the present invention are essential in the production of highly unsaturated polyunsaturated fatty acids having a length greater than 20 and 18 carbons, respectively. The nucleotide sequence of the isolated Thraustochytrium aureum  $\Delta$ 5-desaturase gene (ATCC 34304) is shown in Figure 6, and the amino acid sequence of the corresponding purified protein is shown in Figure 7. The nucleotide sequence of the isolated Saprolegnia diclina  $\Delta$ 5-desaturase gene is shown in Figure 4, and the amino acid sequence of the corresponding purified protein is shown in Figure 5. The nucleotide sequence of the isolated Saprolegnia diclina  $\Delta$ 6-desaturase gene is shown in Figure 2, and the amino acid sequence of the corresponding purified protein is shown in Figure 3. The nucleotide sequence of the isolated Thraustochytrium aureum (BICC7091)  $\Delta$ 5-desaturase gene is shown in Figure 8, and the amino acid sequence of the corresponding purified protein is shown in Figure 9. The nucleotide sequence of the isolated Thraustochytrium aureum  $\Delta$ 6-desaturase gene is shown in Figure 10, and the amino acid sequence of the corresponding purified protein is shown in Figure 11. Finally, the nucleotide sequence of the isolated Isochrysis galbana  $\Delta$ 5-desaturase gene is shown in Figure 14, and the amino acid

sequence of the corresponding purified protein is shown in Figure 15.

As an example of the importance of the genes of the present invention, the isolated  $\Delta 5$ -desaturase genes convert DGLA to AA or convert eicosatetraenoic acid to EPA. AA, for example, cannot be synthesized without the  $\Delta 5$ -desaturase genes and enzymes encoded thereby. The isolated  $\Delta 6$ -desaturase gene of the present invention converts, for example, linoleic acid (18:2n-6) to  $\gamma$ -linoleic acid (GLA) and  $\gamma$ -linolenic acid (GLA) to stearidonic acid (STA).

It should be noted that the present invention also encompasses nucleotide sequences (and the corresponding encoded proteins) having sequences comprising or complementary to at least about 50%, preferably at least about 60%, more preferably at least about 70%, even more preferably at least about 80%, and most preferably at least about 90% of the nucleotides in sequence (i.e., having sequence identity) to SEQ ID NO:19 (i.e., the nucleotide sequence of the  $\Delta 5$ -desaturase gene of Saprolegnia diclina), SEQ ID NO:13 (i.e., the nucleotide sequence of the  $\Delta 6$ -desaturase gene of Thraustochytrium aureum), SEQ ID NO:28 (i.e., the nucleotide sequence of the  $\Delta 5$ -desaturase gene of Thraustochytrium aureum (ATCC 34304), SEQ ID NO:30 (i.e., the nucleotide sequence of the  $\Delta 5$ -desaturase gene of Thraustochytrium aureum (BICC7091), SEQ ID NO:32 (i.e.,  $\Delta 6$ -desaturase gene of Thraustochytrium aureum) and SEQ ID NO:34 (i.e.,  $\Delta 5$ -desaturase gene of Isochrysis galbana), described herein. (All integers between 50% and 100% are also considered to be within the scope of the present invention with respect to percent identity.) Such sequences may be derived from human sources as well as other non-human sources (e.g., C. elegans or mouse).

Furthermore, the present invention also encompasses fragments and derivatives of the nucleotide sequences of the

present invention (i.e., SEQ ID NO:13, SEQ ID NO:19, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32 and SEQ ID NO:34), as well as of the sequences derived from other sources, and having the above-described complementarity or correspondence. Functional equivalents of the above-sequences (i.e., sequences having  $\Delta 5$ -desaturase activity or  $\Delta 6$ -desaturase activity, as appropriate) are also encompassed by the present invention.

The invention also includes a purified polypeptide which desaturates polyunsaturated fatty acids at the carbon 5 position or carbon 6 position and has at least about 50% amino acid similarity or identity, preferably at least about 60% similarity or identity, more preferably at least about 70% similarity or identity, even more preferably at least about 80% similarity or identity, and most preferably at least about 90% similarity or identity to the amino acid sequences (i.e., SEQ ID NO:14 (shown in Figure 3), SEQ ID NO:20 (shown in Figure 5), SEQ ID NO:29 (shown in Figure 7), SEQ ID NO:31 (shown in Figure 9), SEQ ID NO:33 (shown in Figure 11) and SEQ ID NO:35 (shown in Figure 15)) of the above-noted proteins which are, in turn, encoded by the above-described nucleotide sequences. All integers between 50-100% similarity or identity are also included within the scope of the invention.

The term "identity" refers to the relatedness of two sequences on a nucleotide-by-nucleotide basis over a particular comparison window or segment. Thus, identity is defined as the degree of sameness, correspondence or equivalence between the same strands (either sense or antisense) of two DNA segments. "Percentage of sequence identity" is calculated by comparing two optimally aligned sequences over a particular region, determining the number of positions at which the identical base occurs in both sequence in order to yield the number of matched positions, dividing the number of such positions by the total number of positions in the segment being compared and multiplying the result by 100. Optimal alignment of sequences



may be conducted by the algorithm of Smith & Waterman, Appl. Math. 2:482 (1981), by the algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the method of Pearson & Lipman, Proc. Natl. Acad. Sci. (USA) 85:2444 (1988) and by computer  
5 programs which implement the relevant algorithms (e.g., Clustal Macaw Pileup (<http://cmgm.stanford.edu/biochem218/11Multiple.pdf>; Higgins et al., CABIOS. 5L151-153 (1989)), FASTDB (Intelligenetics), BLAST (National Center for Biomedical Information; Altschul et al., Nucleic Acids Research  
10 25:3389-3402 (1997)), PILEUP (Genetics Computer Group, Madison, WI) or GAP, BESTFIT, FASTA and TFASTA (Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, Madison, WI). (See U.S. Patent No. 5,912,120.)

For purposes of the present invention, "complementarity is  
15 defined as the degree of relatedness between two DNA segments. It is determined by measuring the ability of the sense strand of one DNA segment to hybridize with the antisense strand of the other DNA segment, under appropriate conditions, to form a double helix. In the double helix, adenine appears in one  
20 strand, thymine appears in the other strand. Similarly, wherever guanine is found in one strand, cytosine is found in the other. The greater the relatedness between the nucleotide sequences of two DNA segments, the greater the ability to form hybrid duplexes between the strands of the two DNA segments.

25 "Similarity" between two amino acid sequences is defined as the presence of a series of identical as well as conserved amino acid residues in both sequences. The higher the degree of similarity between two amino acid sequences, the higher the correspondence, sameness or equivalence of the two sequences.  
30 ("Identity between two amino acid sequences is defined as the presence of a series of exactly alike or invariant amino acid residues in both sequences.) The definitions of "complementarity", "identity" and "similarity" are well known to those of ordinary skill in the art.



"Encoded by" refers to a nucleic acid sequence which codes for a polypeptide sequence, wherein the polypeptide sequence or a portion thereof contains an amino acid sequence of at least 3 amino acids, more preferably at least 8 amino acids, and even  
5 more preferably at least 15 amino acids from a polypeptide encoded by the nucleic acid sequence.

The present invention also encompasses an isolated nucleotide sequence which encodes PUFA desaturase activity and that is hybridizable, under moderately stringent conditions, to  
10 a nucleic acid having a nucleotide sequence comprising or complementary to the nucleotide sequence comprising SEQ ID NO:13 (shown in Figure 2), SEQ ID NO:19 (shown in Figure 4), SEQ ID NO:28 (shown in Figure 6), SEQ ID NO:30 (shown in Figure 8), SEQ ID NO:32 (shown in Figure 10) or SEQ ID NO:34 (shown in  
15 Figure 14) . A nucleic acid molecule is "hybridizable" to another nucleic acid molecule when a single-stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and ionic strength (see Sambrook et al., "Molecular Cloning: A  
20 Laboratory Manual, Second Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York)). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. "Hybridization" requires that two nucleic acids contain complementary sequences.  
25 However, depending on the stringency of the hybridization, mismatches between bases may occur. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation. Such variables are well known in the art. More specifically, the  
30 greater the degree of similarity or homology between two nucleotide sequences, the greater the value of  $T_m$  for hybrids of nucleic acids having those sequences. For hybrids of

greater than 100 nucleotides in length, equations for calculating  $T_m$  have been derived (see Sambrook et al., *supra*). For hybridization with shorter nucleic acids, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook et al., *supra*).

As used herein, an "isolated nucleic acid fragment or sequence" is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA. (A "fragment" of a specified polynucleotide refers to a polynucleotide sequence which comprises a contiguous sequence of approximately at least about 6 nucleotides, preferably at least about 8 nucleotides, more preferably at least about 10 nucleotides, and even more preferably at least about 15 nucleotides, and most preferable at least about 25 nucleotides identical or complementary to a region of the specified nucleotide sequence.) Nucleotides (usually found in their 5'-monophosphate form) are referred to by their single letter designation as follows: "A" for adenylate or deoxyadenylate (for RNA or DNA, respectively), "C" for cytidylate or deoxycytidylate, "G" for guanylate or deoxyguanylate, "U" for uridylate, "T" for deoxythymidylate, "R" for purines (A or G), "Y" for pyrimidines (C or T), "K" for G or T, "H" for A or C or T, "I" for inosine, and "N" for any nucleotide.

The terms "fragment or subfragment that is functionally equivalent" and "functionally equivalent fragment or subfragment" are used interchangeably herein. These terms refer to a portion or subsequence of an isolated nucleic acid

fragment in which the ability to alter gene expression or produce a certain phenotype is retained whether or not the fragment or subfragment encodes an active enzyme. For example, the fragment or subfragment can be used in the design of chimeric constructs to produce the desired phenotype in a transformed plant. Chimeric constructs can be designed for use in co-suppression or antisense by linking a nucleic acid fragment or subfragment thereof, whether or not it encodes an active enzyme, in the appropriate orientation relative to a plant promoter sequence.

The terms ``homology'', ``homologous'', ``substantially similar'' and ``corresponding substantially'' are used interchangeably herein. They refer to nucleic acid fragments wherein changes in one or more nucleotide bases does not affect the ability of the nucleic acid fragment to mediate gene expression or produce a certain phenotype. These terms also refer to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially alter the functional properties of the resulting nucleic acid fragment relative to the initial, unmodified fragment. It is therefore understood, as those skilled in the art will appreciate, that the invention encompasses more than the specific exemplary sequences.

``Gene'' refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence.

``Native gene'' refers to a gene as found in nature with its own regulatory sequences. In contrast, ``chimeric construct'' refers to a combination of nucleic acid fragments that are not normally found together in nature. Accordingly, a chimeric construct may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that normally

found in nature. (The term ``isolated'' means that the sequence is removed from its natural environment.)

A ``foreign'' gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric constructs. A ``transgene'' is a gene that has been introduced into the genome by a transformation procedure.

``Coding sequence'' refers to a DNA sequence that codes for a specific amino acid sequence. ``Regulatory sequences'' refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include, but are not limited to, promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

``Promoter'' refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an ``enhancer'' is a DNA sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoter sequences can also be located within the transcribed portions of genes, and/or downstream of the transcribed sequences. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as ``constitutive promoters''. New promoters of various types useful in plant cells are constantly being discovered; numerous

examples may be found in the compilation by Okamuro and Goldberg, (1989) *Biochemistry of Plants* 15:1-82. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA  
5 fragments of some variation may have identical promoter activity.

An ``intron'' is an intervening sequence in a gene that does not encode a portion of the protein sequence. Thus, such sequences are transcribed into RNA but are then excised and are  
10 not translated. The term is also used for the excised RNA sequences. An ``exon'' is a portion of the sequence of a gene that is transcribed and is found in the mature messenger RNA derived from the gene, but is not necessarily a part of the sequence that encodes the final gene product.

The ``translation leader sequence'' refers to a DNA  
15 sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect  
20 processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner, R. and Foster, G. D. (1995) *Molecular Biotechnology* 3:225).

The ``3' non-coding sequences'' refer to DNA sequences  
25 located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic  
30 acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al., (1989) *Plant Cell* 1:671-680.

``RNA transcript'' refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When  
35 the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from post-transcriptional processing of the primary transcript and is referred to as the mature RNA.



``Messenger RNA (mRNA)'' refers to the RNA that is without introns and that can be translated into protein by the cell.

``cDNA'' refers to a DNA that is complementary to and synthesized from a mRNA template using the enzyme reverse

5 transcriptase. The cDNA can be single-stranded or converted into the double-stranded form using the Klenow fragment of DNA polymerase I. ``Sense'' RNA refers to RNA transcript that includes the mRNA and can be translated into protein within a cell or *in vitro*. ``Antisense RNA'' refers to an RNA  
10 transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene (U.S. Patent No. 5,107,065). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding  
15 sequence, introns, or the coding sequence. ``Functional RNA'' refers to antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes. The terms ``complement'' and ``reverse complement'' are used interchangeably herein with respect to mRNA transcripts, and  
20 are meant to define the antisense RNA of the message.

The term ``endogenous RNA'' refers to any RNA which is encoded by any nucleic acid sequence present in the genome of the host prior to transformation with the recombinant construct of the present invention, whether naturally-occurring or non-  
25 naturally occurring, i.e., introduced by recombinant means, mutagenesis, etc.

The term ``non-naturally occurring'' means artificial, not consistent with what is normally found in nature.

The term ``operably linked'' refers to the association of  
30 nucleic acid sequences on a single nucleic acid fragment so that the function of one is regulated by the other. For example, a promoter is operably linked with a coding sequence when it is capable of regulating the expression of that coding sequence (i.e., that the coding sequence is under the  
35 transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in a sense or antisense orientation. In another example, the complementary RNA regions of the invention can be operably linked, either



directly or indirectly, 5' to the target mRNA, or 3' to the target mRNA, or within the target mRNA, or a first complementary region is 5' and its complement is 3' to the target mRNA.

5       The term ``expression'', as used herein, refers to the production of a functional end-product. Expression of a gene involves transcription of the gene and translation of the mRNA into a precursor or mature protein. ``Antisense inhibition'' refers to the production of antisense RNA transcripts capable  
10 of suppressing the expression of the target protein. ``Co-suppression'' refers to the production of sense RNA transcripts capable of suppressing the expression of identical or substantially similar foreign or endogenous genes (U.S. Patent No. 5,231,020).

15       ``Mature'' protein refers to a post-translationally processed polypeptide; i.e., one from which any pre- or propeptides present in the primary translation product have been removed. ``Precursor'' protein refers to the primary product of translation of mRNA; i.e., with pre- and propeptides  
20 still present. Pre- and propeptides may be but are not limited to intracellular localization signals.

      ``Stable transformation'' refers to the transfer of a nucleic acid fragment into a genome of a host organism, including both nuclear and organellar genomes, resulting in  
25 genetically stable inheritance. In contrast, ``transient transformation'' refers to the transfer of a nucleic acid fragment into the nucleus, or DNA-containing organelle, of a host organism resulting in gene expression without integration or stable inheritance. Host organisms containing the  
30 transformed nucleic acid fragments are referred to as ``transgenic'' organisms. The preferred method of cell transformation of rice, corn and other monocots is the use of particle-accelerated or ``gene gun'' transformation technology (Klein et al., (1987) *Nature (London)* 327:70-73; U.S. Patent  
35 No. 4,945,050), or an Agrobacterium-mediated method using an appropriate Ti plasmid containing the transgene (Ishida Y. et al., 1996, *Nature Biotech.* 14:745-750). The term

``transformation'' as used herein refers to both stable transformation and transient transformation.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J., Fritsch, E.F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989 (hereinafter ``Sambrook``).

The term ``recombinant'' refers to an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the manipulation of isolated segments of nucleic acids by genetic engineering techniques.

``PCR'' or ``Polymerase Chain Reaction'' is a technique for the synthesis of large quantities of specific DNA segments, consists of a series of repetitive cycles (Perkin Elmer Cetus Instruments, Norwalk, CT). Typically, the double stranded DNA is heat denatured, the two primers complementary to the 3' boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature. One set of these three consecutive steps is referred to as a cycle.

Polymerase chain reaction (``PCR'') is a powerful technique used to amplify DNA millions of fold, by repeated replication of a template, in a short period of time. (Mullis et al, *Cold Spring Harbor Symp. Quant. Biol.* 51:263-273 (1986); Erlich et al, European Patent Application 50,424; European Patent Application 84,796; European Patent Application 258,017, European Patent Application 237,362; Mullis, European Patent Application 201,184, Mullis et al U.S. Patent No. 4,683,202; Erlich, U.S. Patent No. 4,582,788; and Saiki et al, U.S. Patent No. 4,683,194). The process utilizes sets of specific in vitro synthesized oligonucleotides to prime DNA synthesis. The design of the primers is dependent upon the sequences of DNA that are desired to be analyzed. The technique is carried out through many cycles (usually 20-50) of melting the template at high temperature, allowing the primers to anneal to complementary sequences within the template and then replicating the template with DNA polymerase.

The products of PCR reactions are analyzed by separation in agarose gels followed by ethidium bromide staining and visualization with UV transillumination. Alternatively, radioactive dNTPs can be added to the PCR in order to incorporate label into the products. In this case the products of PCR are visualized by exposure of the gel to x-ray film. The added advantage of radiolabeling PCR products is that the levels of individual amplification products can be quantitated.

The terms ``recombinant construct'', ``expression construct'' and ``recombinant expression construct'' are used interchangeably herein. These terms refer to a functional unit of genetic material that can be inserted into the genome of a cell using standard methodology well known to one skilled in the art. Such construct may be itself or may be used in conjunction with a vector. If a vector is used then the choice of vector is dependent upon the method that will be used to transform host plants as is well known to those skilled in the art. For example, a plasmid vector can be used. The skilled artisan is well aware of the genetic elements that must be present on the vector in order to successfully transform, select and propagate host cells comprising any of the isolated nucleic acid fragments of the invention. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al., (1985) *EMBO J.* 4:2411-2418; De Almeida et al., (1989) *Mol. Gen. Genetics* 218:78-86), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, Western analysis of protein expression, or phenotypic analysis.

#### Production of the $\Delta 5$ -Desaturase Enzymes and the $\Delta 6$ -Desaturase Enzymes

Once the gene encoding any one of the desaturase enzymes has been isolated, it may then be introduced into either a prokaryotic or eukaryotic host cell through the use of a vector

or construct. The vector, for example, a bacteriophage, cosmid or plasmid, may comprise the nucleotide sequence encoding either of the  $\Delta 5$ -desaturase enzymes, or the  $\Delta 6$ -desaturase enzyme, as well as any regulatory sequence (e.g., promoter) which is functional in the host cell and is able to elicit expression of the desaturase encoded by the nucleotide sequence. The regulatory sequence is in operable association with or operably linked to the nucleotide sequence. (As noted above, regulatory is said to be ``operably linked'' with a coding sequence if the regulatory sequence affects transcription or expression of the coding sequence.) Suitable promoters include, for example, those from genes encoding alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglucosomerase, phosphoglycerate kinase, acid phosphatase, T7, TPI, lactase, metallothionein, cytomegalovirus immediate early, whey acidic protein, glucoamylase, and promoters activated in the presence of galactose, for example, GAL1 and GAL10. Additionally, nucleotide sequences which encode other proteins, oligosaccharides, lipids, etc. may also be included within the vector as well as other regulatory sequences such as a polyadenylation signal (e.g., the poly-A signal of SV-40T-antigen, ovalalbumin or bovine growth hormone). The choice of sequences present in the construct is dependent upon the desired expression products as well as the nature of the host cell.

As noted above, once the vector has been constructed, it may then be introduced into the host cell of choice by methods known to those of ordinary skill in the art including, for example, transfection, transformation and electroporation (see Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> ed., Vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press (1989)).

The host cell is then cultured under suitable conditions permitting expression of the genes leading to the production of the desired PUFA, which is then recovered and purified.

Examples of suitable prokaryotic host cells include, for example, bacteria such as Escherichia coli, Bacillus subtilis as well as cyanobacteria such as Spirulina spp. (i.e., blue-green algae). Examples of suitable eukaryotic host cells include, for example, mammalian cells, plant cells, yeast cells such as Saccharomyces cerevisiae, Saccharomyces carlsbergensis,  
10 Lipomyces starkey, Candida spp. such as Yarrowia (Candida) lipolytica, Kluyveromyces spp., Pichia spp., Trichoderma spp. or Hansenula spp., or fungal cells such as filamentous fungal cells, for example, Aspergillus, Neurospora and Penicillium. Preferably, Saccharomyces cerevisiae (baker's yeast) cells are  
15 utilized.

Expression in a host cell can be accomplished in a transient or stable fashion. Transient expression can occur from introduced constructs which contain expression signals functional in the host cell, but which constructs do not  
20 replicate and rarely integrate in the host cell, or where the host cell is not proliferating. Transient expression also can be accomplished by inducing the activity of a regulatable promoter operably linked to the gene of interest, although such inducible systems frequently exhibit a low basal level of  
25 expression. Stable expression can be achieved by introduction of a construct that can integrate into the host genome or that autonomously replicates in the host cell. Stable expression of the gene of interest can be selected for through the use of a selectable marker located on or transfected with the expression  
30 construct, followed by selection for cells expressing the marker. When stable expression results from integration, the site of the construct's integration can occur randomly within



the host genome or can be targeted through the use of constructs containing regions of homology with the host genome sufficient to target recombination with the host locus. Where constructs are targeted to an endogenous locus, all or some of the transcriptional and translational regulatory regions can be provided by the endogenous locus.

A transgenic mammal may also be used in order to express the enzyme(s) of interest (i.e., one or more of the  $\Delta 5$ -desaturases, one or more of the  $\Delta 6$ -desaturases, or a combination thereof), and ultimately the PUFA(s) of interest. More specifically, once the above-described construct is created, it may be inserted into the pronucleus of an embryo. The embryo may then be implanted into a recipient female. Alternatively, a nuclear transfer method could also be utilized (Schnieke et al., Science 278:2130-2133 (1997)). Gestation and birth are then permitted (see, e.g., U.S. Patent No. 5,750,176 and U.S. Patent No. 5,700,671). Milk, tissue or other fluid samples from the offspring should then contain altered levels of PUFAs, as compared to the levels normally found in the non-transgenic animal. Subsequent generations may be monitored for production of the altered or enhanced levels of PUFAs and thus incorporation of the gene encoding the desired desaturase enzyme into their genomes. The mammal utilized as the host may be selected from the group consisting of, for example, a mouse, a rat, a rabbit, a pig, a goat, a sheep, a horse and a cow. However, any mammal may be used provided it has the ability to incorporate DNA encoding the enzyme of interest into its genome.

For expression of a desaturase polypeptide, functional transcriptional and translational initiation and termination regions are operably linked to the DNA encoding the desaturase



polypeptide. Transcriptional and translational initiation and termination regions are derived from a variety of nonexclusive sources, including the DNA to be expressed, genes known or suspected to be capable of expression in the desired system, expression vectors, chemical synthesis, or from an endogenous locus in a host cell. Expression in a plant tissue and/or plant part presents certain efficiencies, particularly where the tissue or part is one which is harvested early, such as seed, leaves, fruits, flowers, roots, etc. Expression can be targeted to that location with the plant by utilizing specific regulatory sequence such as those of U.S. Patent Nos. 5,463,174, 4,943,674, 5,106,739, 5,175,095, 5,420,034, 5,188,958, and 5,589,379. Alternatively, the expressed protein can be an enzyme which produces a product which may be incorporated, either directly or upon further modifications, into a fluid fraction from the host plant. Expression of a desaturase gene, or antisense desaturase transcripts, can alter the levels of specific PUFAs, or derivatives thereof, found in plant parts and/or plant tissues. The desaturase polypeptide coding region may be expressed either by itself or with other genes, in order to produce tissues and/or plant parts containing higher proportions of desired PUFAs or in which the PUFA composition more closely resembles that of human breast milk (Prieto et al., PCT publication WO 95/24494). The termination region may be derived from the 3' region of the gene from which the initiation region was obtained or from a different gene. A large number of termination regions are known to and have been found to be satisfactory in a variety of hosts from the same and different genera and species. The termination region usually is selected as a matter of convenience rather than because of any particular property.

As noted above, a plant (e.g., Glycine max (soybean) or Brassica napus (canola)) or plant tissue may also be utilized as a host or host cell, respectively, for expression of the desaturase enzyme which may, in turn, be utilized in the production of polyunsaturated fatty acids. More specifically, desired PUFAS can be expressed in seed. Methods of isolating seed oils are known in the art. Thus, in addition to providing a source for PUFAs, seed oil components may be manipulated through the expression of the desaturase gene, as well as perhaps other desaturase genes and elongase genes, in order to provide seed oils that can be added to nutritional compositions, pharmaceutical compositions, animal feeds and cosmetics. Once again, a vector which comprises a DNA sequence encoding the desaturase operably linked to a promoter, will be introduced into the plant tissue or plant for a time and under conditions sufficient for expression of the desaturase gene. The vector may also comprise one or more genes that encode other enzymes, for example,  $\Delta 4$ -desaturase, elongase,  $\Delta 12$ -desaturase,  $\Delta 15$ -desaturase,  $\Delta 17$ -desaturase, and/or  $\Delta 19$ -desaturase. The plant tissue or plant may produce the relevant substrate (e.g., DGLA (in the case of  $\Delta 5$ -desaturase), ALA (in the case of  $\Delta 6$ -desaturase), etc.) upon which the enzymes act or a vector encoding enzymes which produce such substrates may be introduced into the plant tissue, plant cell or plant. In addition, substrate may be sprayed on plant tissues expressing the appropriate enzymes. Using these various techniques, one may produce PUFAs (e.g., n-6 unsaturated fatty acids such as AA, or n-3 fatty acids such as EPA or STA) by use of a plant cell, plant tissue or plant. It should also be noted that the invention also encompasses a transgenic plant comprising the above-described vector, wherein expression of the nucleotide

sequence of the vector results in production of a polyunsaturated fatty acid in, for example, the seeds of the transgenic plant.

The regeneration, development, and cultivation of plants from single plant protoplast transformants or from various transformed explants is well known in the art (Weissbach and Weissbach, In: Methods for Plant Molecular Biology, (Eds.), Academic Press, Inc. San Diego, CA, (1988)). This regeneration and growth process typically includes the steps of selection of transformed cells, culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil.

The development or regeneration of plants containing the foreign, exogenous gene that encodes a protein of interest is well known in the art. Preferably, the regenerated plants are self-pollinated to provide homozygous transgenic plants. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important lines. Conversely, pollen from plants of these important lines is used to pollinate regenerated plants. A transgenic plant of the present invention containing a desired polypeptide is cultivated using methods well known to one skilled in the art.

There are a variety of methods for the regeneration of plants from plant tissue. The particular method of regeneration will depend on the starting plant tissue and the particular plant species to be regenerated.

Methods for transforming dicots, primarily by use of *Agrobacterium tumefaciens*, and obtaining transgenic plants have been published for cotton (U.S. Patent No. 5,004,863, U.S. Patent No. 5,159,135, U.S. Patent No. 5,518,908); soybean (U.S. Patent No. 5,569,834, U.S. Patent No. 5,416,011, McCabe et. al., *BiolTechnology* 6:923 (1988), Christou et al., *Plant Physiol.* 87:671-674 (1988)); *Brassica* (U.S. Patent No. 5,463,174); peanut (Cheng et al., *Plant Cell Rep.*

15:653-657 (1996), McKently et al., *Plant Cell Rep.* 14:699-703 (1995)); papaya; and pea (Grant et al., *Plant Cell Rep.* 15:254-258, (1995)).

Transformation of monocotyledons using electroporation,  
5 particle bombardment, and *Agrobacterium* have also been reported. Transformation and plant regeneration have been achieved in asparagus (Bytebier et al., *Proc. Natl. Acad. Sci. (USA)* 84:5354, (1987)); barley (Wan and Lemaux, *Plant Physiol* 104:37 (1994)); *Zea mays* (Rhodes et al., *Science* 240:204  
10 (1988), Gordon-Kamm et al., *Plant Cell* 2:603-618 (1990), Fromm et al., *BiolTechnology* 8:833 (1990), Koziel et al., *BiolTechnology* 11: 194, (1993), Armstrong et al., *Crop Science* 35:550-557 (1995)); oat (Somers et al., *BiolTechnology* 10: 15  
89 (1992)); orchard grass (Horn et al., *Plant Cell Rep.* 7:469  
15 (1988)); rice (Toriyama et al., *TheorAppl. Genet.* 205:34, (1986); Part et al., *Plant Mol. Biol.* 32:1135-1148, (1996); Abedinia et al., *Aust. J. Plant Physiol.* 24:133-141 (1997); Zhang and Wu, *Theor. Appl. Genet.* 76:835 (1988); Zhang et al. *Plant Cell Rep.* 7:379, (1988); Battraw and Hall, *Plant Sci.*  
20 86:191-202 (1992); Christou et al., *Bio/Technology* 9:957 (1991)); rye (De la Pena et al., *Nature* 325:274 (1987)); sugarcane (Bower and Birch, *Plant J.* 2:409 (1992)); tall fescue (Wang et al., *BiolTechnology* 10:691 (1992)), and wheat (Vasil et al., *Bio/Technology* 10:667 (1992); U.S. Patent  
25 No. 5,631,152).

Assays for gene expression based on the transient expression of cloned nucleic acid constructs have been developed by introducing the nucleic acid molecules into plant cells by polyethylene glycol treatment, electroporation, or  
30 particle bombardment (Marcotte et al., *Nature* 335:454-457 (1988); Marcotte et al., *Plant Cell* 1:523-532 (1989); McCarty et al., *Cell* 66:895-905 (1991); Hattori et al., *Genes Dev.* 6:609-618 (1992); Goff et al., *EMBO J.* 9:2517-2522 (1990)).

Transient expression systems may be used to functionally  
35 dissect gene constructs (see generally, Maliga et al., *Methods in Plant Molecular Biology*, Cold Spring Harbor Press (1995)).

It is understood that any of the nucleic acid molecules of the present invention can be introduced into a plant cell in a permanent or transient manner in combination with other genetic elements such as vectors, promoters, enhancers etc.

5        In addition to the above discussed procedures, practitioners are familiar with the standard resource materials which describe specific conditions and procedures for the construction, manipulation and isolation of macromolecules (e.g., DNA molecules, plasmids, etc.), generation of  
10 recombinant organisms and the screening and isolating of clones, (see for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989); Maliga et al., Methods in Plant Molecular Biology, Cold Spring Harbor Press (1995); Birren et al., Genome Analysis: Detecting Genes,  
15 1, Cold Spring Harbor, New York (1998); Birren et al., Genome Analysis: Analyzing DNA, 2, Cold Spring Harbor, New York (1998); Plant Molecular Biology: A Laboratory Manual, eds. Clark, Springer, New York (1997)).

20        The substrates which may be produced by the host cell either naturally or transgenically, as well as the enzymes which may be encoded by DNA sequences present in the vector which is subsequently introduced into the host cell, are shown in Figure 1.

25        In view of the above, the present invention encompasses a method of producing the desaturase enzymes (i.e.,  $\Delta 5$  or  $\Delta 6$ ) comprising the steps of: 1) isolating the nucleotide sequence of the gene encoding the desaturase enzyme; 2) constructing a vector comprising said nucleotide sequence; and 3) introducing said vector into a host cell under time and conditions  
30 sufficient for the production of the desaturase enzyme.

      The present invention also encompasses a method of producing polyunsaturated fatty acids comprising exposing an



acid to the enzyme such that the desaturase converts the acid to a polyunsaturated fatty acid. For example, when 20:3n-6 is exposed to a  $\Delta$ 5-desaturase enzyme, it is converted to AA. AA may then be exposed to elongase which elongates the AA to  
5 adrenic acid (i.e., 22:4n-6). Alternatively,  $\Delta$ 5-desaturase may be utilized to convert 20:4n-3 to 20:5n-3 which may be exposed to elongase and converted to (n-3)-docosapentaenoic acid. The (n-3)-docosapentaenoic acid may then be converted to DHA by use of  $\Delta$ 4-desaturase. Thus,  $\Delta$ 5-desaturase may be used in the  
10 production of polyunsaturated fatty acids which may be used, in turn, for particular beneficial purposes.

With respect to the role of  $\Delta$ 6-desaturase, linoleic acid may be exposed to the enzyme such that the enzyme converts the acid to GLA. An elongase may then be used to convert the GLA  
15 to DGLA. The DGLA then may be converted to AA by exposing the DGLA to a  $\Delta$ 5-desaturase. As another example, ALA may be exposed to a  $\Delta$ 6-desaturase in order to convert the ALA to STA. The STA may then be converted to 20:4n-3 by using an elongase. Subsequently, the 20:4n-3 may be converted to EPA by exposing  
20 the 20:4n-3 to a  $\Delta$ 5-desaturase. Thus, the  $\Delta$ 6-desaturase may be used in the production of PUFAs which have may advantageous properties or may be used in the production of other PUFAs.

#### 25 Uses of the $\Delta$ 5-Desaturases Genes, the $\Delta$ 6-Desaturase Genes, and Enzymes Encoded Thereby

As noted above, the isolated desaturase genes and the desaturase enzymes encoded thereby have many uses. For example, the gene and corresponding enzyme may be used  
30 indirectly or directly in the production of polyunsaturated fatty acids, for example,  $\Delta$ 5-desaturase may be used in the

production of AA, adrenic acid or EPA. Delta-6 desaturase may be used either indirectly or directly in the production of GLA, DGLA, STA or 20:4n-3. ("Directly" is meant to encompass the situation where the enzyme directly converts the acid to another acid, the latter of which is utilized in a composition (e.g., the conversion of DGLA to AA). "Indirectly" is meant to encompass the situation where an acid is converted to another acid (i.e., a pathway intermediate) by the desaturase (e.g., DGLA to AA) and then the latter acid is converted to another acid by use of a non-desaturase enzyme (e.g., AA to adrenic acid by elongase or by use of another desaturase enzyme (e.g., AA to EPA by  $\Delta 17$ -desaturase.)). These polyunsaturated fatty acids (i.e., those produced either directly or indirectly by activity of the desaturase enzyme) may be added to, for example, nutritional compositions, pharmaceutical compositions, cosmetics, and animal feeds, all of which are encompassed by the present invention. These uses are described, in detail, below.

#### Nutritional Compositions

The present invention includes nutritional compositions. Such compositions, for purposes of the present invention, include any food or preparation for human consumption including for enteral or parenteral consumption, which when taken into the body (a) serve to nourish or build up tissues or supply energy and/or (b) maintain, restore or support adequate nutritional status or metabolic function.

The nutritional composition of the present invention comprises at least one oil or acid produced directly or indirectly by use of the desaturase gene, in accordance with the present invention, and may either be in a solid or liquid form. Additionally, the composition may include edible

macronutrients, vitamins and minerals in amounts desired for a particular use. The amount of such ingredients will vary depending on whether the composition is intended for use with normal, healthy infants, children or adults having specialized  
5 needs such as those which accompany certain metabolic conditions (e.g., metabolic disorders).

Examples of macronutrients which may be added to the composition include but are not limited to edible fats, carbohydrates and proteins. Examples of such edible fats  
10 include but are not limited to coconut oil, soy oil, and mono- and diglycerides. Examples of such carbohydrates include but are not limited to glucose, edible lactose and hydrolyzed starch. Additionally, examples of proteins which may be utilized in the nutritional composition of the invention  
15 include but are not limited to soy proteins, electrodialysed whey, electrodialysed skim milk, milk whey, or the hydrolysates of these proteins.

With respect to vitamins and minerals, the following may be added to the nutritional compositions of the present  
20 invention: calcium, phosphorus, potassium, sodium, chloride, magnesium, manganese, iron, copper, zinc, selenium, iodine, and Vitamins A, E, D, C, and the B complex. Other such vitamins and minerals may also be added.

The components utilized in the nutritional compositions of  
25 the present invention will be of semi-purified or purified origin. By semi-purified or purified is meant a material which has been prepared by purification of a natural material or by synthesis.

Examples of nutritional compositions of the present  
30 invention include but are not limited to infant formulas, dietary supplements, dietary substitutes, and rehydration compositions. Nutritional compositions of particular interest

include but are not limited to those utilized for enteral and parenteral supplementation for infants, specialist infant formulas, supplements for the elderly, and supplements for those with gastrointestinal difficulties and/or malabsorption.

5       The nutritional composition of the present invention may also be added to food even when supplementation of the diet is not required. For example, the composition may be added to food of any type including but not limited to margarines, modified butters, cheeses, milk, yogurt, chocolate, candy,  
10   snacks, salad oils, cooking oils, cooking fats, meats, fish and beverages.

      In a preferred embodiment of the present invention, the nutritional composition is an enteral nutritional product, more preferably, an adult or pediatric enteral nutritional product.  
15   This composition may be administered to adults or children experiencing stress or having specialized needs due to chronic or acute disease states. The composition may comprise, in addition to polyunsaturated fatty acids produced in accordance with the present invention, macronutrients, vitamins and  
20   minerals as described above. The macronutrients may be present in amounts equivalent to those present in human milk or on an energy basis, i.e., on a per calorie basis.

      Methods for formulating liquid or solid enteral and parenteral nutritional formulas are well known in the art.  
25   (See also the Examples below.)

      The enteral formula, for example, may be sterilized and subsequently utilized on a ready-to-feed (RTF) basis or stored in a concentrated liquid or powder. The powder can be prepared by spray drying the formula prepared as indicated above, and  
30   reconstituting it by rehydrating the concentrate. Adult and pediatric nutritional formulas are well known in the art and are commercially available (e.g., Similac®, Ensure®, Jevity®

and Alimentum® from Ross Products Division, Abbott Laboratories, Columbus, Ohio). An oil or acid produced in accordance with the present invention may be added to any of these formulas.

5       The energy density of the nutritional compositions of the present invention, when in liquid form, may range from about 0.6 Kcal to about 3 Kcal per ml. When in solid or powdered form, the nutritional supplements may contain from about 1.2 to more than 9 Kcals per gram, preferably about 3 to 7 Kcals per  
10 gm. In general, the osmolality of a liquid product should be less than 700 mOsm and, more preferably, less than 660 mOsm.

      The nutritional formula may include macronutrients, vitamins, and minerals, as noted above, in addition to the PUFAs produced in accordance with the present invention. The  
15 presence of these additional components helps the individual ingest the minimum daily requirements of these elements. In addition to the provision of PUFAs, it may also be desirable to add zinc, copper, folic acid and antioxidants to the composition. It is believed that these substance boost a  
20 stressed immune system and will therefore provide further benefits to the individual receiving the composition. A pharmaceutical composition may also be supplemented with these elements.

      In a more preferred embodiment, the nutritional  
25 composition comprises, in addition to antioxidants and at least one PUFA, a source of carbohydrate wherein at least 5 weight percent of the carbohydrate is indigestible oligosaccharide. In a more preferred embodiment, the nutritional composition additionally comprises protein, taurine, and carnitine.

30       As noted above, the PUFAs produced in accordance with the present invention, or derivatives thereof, may be added to a dietary substitute or supplement, particularly an infant



formula, for patients undergoing intravenous feeding or for preventing or treating malnutrition or other conditions or disease states. As background, it should be noted that human breast milk has a fatty acid profile comprising from about 5 0.15% to about 0.36% as DHA, from about 0.03% to about 0.13% as EPA, from about 0.30% to about 0.88% as AA, from about 0.22% to about 0.67% as DGLA, and from about 0.27% to about 1.04% as GLA. Thus, fatty acids such as AA, EPA and/or docosahexaenoic acid (DHA), produced in accordance with the present invention, 10 can be used to alter, for example, the composition of infant formulas in order to better replicate the PUFA content of human breast milk or to alter the presence of PUFAs normally found in a non-human mammal's milk. In particular, a composition for use in a pharmacologic or food supplement, particularly a 15 breast milk substitute or supplement, will preferably comprise one or more of AA, DGLA and GLA. More preferably, the oil will comprise from about 0.3 to 30% AA, from about 0.2 to 30% DGLA, and/or from about 0.2 to about 30% GLA.

Parenteral nutritional compositions comprising from about 20 2 to about 30 weight percent fatty acids calculated as triglycerides are encompassed by the present invention. The preferred composition has about 1 to about 25 weight percent of the total PUFA composition as GLA (U.S. Patent No. 5,196,198). Other vitamins, particularly fat-soluble vitamins such as 25 vitamin A, D, E and L-carnitine can optionally be included. When desired, a preservative such as alpha-tocopherol may be added in an amount of about 0.1% by weight.

In addition, the ratios of AA, DGLA and GLA can be adapted for a particular given end use. When formulated as a breast 30 milk supplement or substitute, a composition which comprises one or more of AA, DGLA and GLA will be provided in a ratio of about 1:19:30 to about 6:1:0.2, respectively. For example, the

breast milk of animals can vary in ratios of AA:DGLA:GLA ranging from 1:19:30 to 6:1:0.2, which includes intermediate ratios which are preferably about 1:1:1, 1:2:1, 1:1:4. When produced together in a host cell, adjusting the rate and percent of conversion of a precursor substrate such as GLA and DGLA to AA can be used to precisely control the PUFA ratios. For example, a 5% to 10% conversion rate of DGLA to AA can be used to produce an AA to DGLA ratio of about 1:19, whereas a conversion rate of about 75% TO 80% can be used to produce an AA to DGLA ratio of about 6:1. Therefore, whether in a cell culture system or in a host animal, regulating the timing, extent and specificity of desaturase expression, as well as the expression of other desaturases and elongases, can be used to modulate PUFA levels and ratios. The PUFAs/acids produced in accordance with the present invention (e.g., AA and EPA) may then be combined with other PUFAs/acids (e.g., GLA) in the desired concentrations and ratios.

Additionally, PUFA produced in accordance with the present invention or host cells containing them may also be used as animal food supplements to alter an animal's tissue or milk fatty acid composition to one more desirable for human or animal consumption.

#### Pharmaceutical Compositions

The present invention also encompasses a pharmaceutical composition comprising one or more of the acids and/or resulting oils produced using the desaturase genes described herein, in accordance with the methods described herein. More specifically, such a pharmaceutical composition may comprise one or more of the acids and/or oils as well as a standard, well-known, non-toxic pharmaceutically acceptable carrier, adjuvant or vehicle such as, for example, phosphate buffered saline, water, ethanol, polyols, vegetable oils, a wetting

agent or an emulsion such as a water/oil emulsion. The composition may be in either a liquid or solid form. For example, the composition may be in the form of a tablet, capsule, ingestible liquid or powder, injectible, or topical  
5 ointment or cream. Proper fluidity can be maintained, for example, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants. It may also be desirable to include isotonic agents, for example, sugars, sodium chloride and the like. Besides such inert  
10 diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening agents, flavoring agents and perfuming agents.

Suspensions, in addition to the active compounds, may comprise suspending agents such as, for example, ethoxylated  
15 isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth or mixtures of these substances.

Solid dosage forms such as tablets and capsules can be  
20 prepared using techniques well known in the art. For example, PUFAs produced in accordance with the present invention can be tableted with conventional tablet bases such as lactose, sucrose, and cornstarch in combination with binders such as acacia, cornstarch or gelatin, disintegrating agents such as  
25 potato starch or alginic acid, and a lubricant such as stearic acid or magnesium stearate. Capsules can be prepared by incorporating these excipients into a gelatin capsule along with antioxidants and the relevant PUFA(s). The antioxidant and PUFA components should fit within the guidelines presented  
30 above.

For intravenous administration, the PUFAs produced in accordance with the present invention or derivatives thereof may be incorporated into commercial formulations such as Intralipids™. The typical normal adult plasma fatty acid

profile comprises 6.64 to 9.46% of AA, 1.45 to 3.11% of DGLA, and 0.02 to 0.08% of GLA. These PUFAs or their metabolic precursors can be administered alone or in combination with other PUFAs in order to achieve a normal fatty acid profile in a patient. Where desired, the individual components of the formulations may be provided individually, in kit form, for single or multiple use. A typical dosage of a particular fatty acid is from 0.1 mg to 20 g (up to 100 g) daily and is preferably from 10 mg to 1, 2, 5 or 10 g daily.

Possible routes of administration of the pharmaceutical compositions of the present invention include, for example, enteral (e.g., oral and rectal) and parenteral. For example, a liquid preparation may be administered, for example, orally or rectally. Additionally, a homogenous mixture can be completely dispersed in water, admixed under sterile conditions with physiologically acceptable diluents, preservatives, buffers or propellants in order to form a spray or inhalant. The route of administration will, of course, depend upon the desired effect. For example, if the composition is being utilized to treat rough, dry, or aging skin, to treat injured or burned skin, or to treat skin or hair affected by a disease or condition, it may perhaps be applied topically.

The dosage of the composition to be administered to the patient may be determined by one of ordinary skill in the art and depends upon various factors such as weight of the patient, age of the patient, immune status of the patient, etc.

With respect to form, the composition may be, for example, a solution, a dispersion, a suspension, an emulsion or a sterile powder which is then reconstituted.

The present invention also includes the treatment of various disorders by use of the pharmaceutical and/or nutritional compositions described herein. In particular, the compositions of the present invention may be used to treat restenosis after angioplasty. Furthermore, symptoms of

inflammation, rheumatoid arthritis, asthma and psoriasis may also be treated with the compositions of the invention.

Evidence also indicates that PUFAs may be involved in calcium metabolism; thus, the compositions of the present invention  
5 may, perhaps, be utilized in the treatment or prevention of osteoporosis and of kidney or urinary tract stones.

Additionally, the compositions of the present invention may also be used in the treatment of cancer. Malignant cells have been shown to have altered fatty acid compositions.

10 Addition of fatty acids has been shown to slow their growth, cause cell death and increase their susceptibility to chemotherapeutic agents. Moreover, the compositions of the present invention may also be useful for treating cachexia associated with cancer.

15 The compositions of the present invention may also be used to treat diabetes (see U.S. Patent No. 4,826,877 and Horrobin et al., Am. J. Clin. Nutr. Vol. 57 (Suppl.) 732S-737S). Altered fatty acid metabolism and composition have been demonstrated in diabetic animals.

20 Furthermore, the compositions of the present invention, comprising PUFAs produced either directly or indirectly through the use of the desaturase enzymes, may also be used in the treatment of eczema, in the reduction of blood pressure, and in the improvement of mathematics examination scores.

25 Additionally, the compositions of the present invention may be used in inhibition of platelet aggregation, induction of vasodilation, reduction in cholesterol levels, inhibition of proliferation of vessel wall smooth muscle and fibrous tissue (Brenner et al., Adv. Exp. Med. Biol. Vol. 83, p.85-101, 1976),  
30 reduction or prevention of gastrointestinal bleeding and other side effects of non-steroidal anti-inflammatory drugs (see U.S. Patent No. 4,666,701), prevention or treatment of endometriosis and premenstrual syndrome (see U.S. Patent No. 4,758,592), and



treatment of myalgic encephalomyelitis and chronic fatigue after viral infections (see U.S. Patent No. 5,116,871).

Further uses of the compositions of the present invention include use in the treatment of AIDS, multiple sclerosis, and  
5 inflammatory skin disorders, as well as for maintenance of general health.

Additionally, the composition of the present invention may be utilized for cosmetic purposes. It may be added to pre-existing cosmetic compositions such that a mixture is formed or  
10 may be used as a sole composition.

#### Veterinary Applications

It should be noted that the above-described pharmaceutical and nutritional compositions may be utilized in connection with  
15 animals (i.e., domestic or non-domestic), as well as humans, as animals experience many of the same needs and conditions as humans. For example, the oil or acids of the present invention may be utilized in animal or aquaculture feed supplements, animal feed substitutes, animal vitamins or in animal topical  
20 ointments.

The present invention may be illustrated by the use of the following non-limiting examples:

#### Example 1

25 Design of Degenerate Oligonucleotides for the Isolation of Desaturases from Fungi and cDNA Library Construction

Analysis of the fatty acid composition of *Saprolegnia diclina* (*S.diclina*) (ATCC 56851) revealed the presence of a  
30 considerable amount of arachidonic acid (ARA, 20:4 n-6) and eicosapentanoic acid (EPA, 20:5 n-3). Thus, it was thought that this organism contained an active  $\Delta$ 6-desaturase capable of converting linoleic acid (LA, 18:2 n-6) to gamma-linolenic acid (GLA, 18:3 n-6), and an active  $\Delta$ 5-desaturase that would convert

dihomo-gamma-linolenic acid (DGLA, 20:3 n-6) to arachidonic acid (ARA, 20:4 n-6) (Figure 1). In addition, it was thought that *S. diclina* also contained a  $\Delta 17$ -desaturase capable of desaturating ARA to EPA.

5       The fatty acid composition analysis of *Thraustochytrium aureum* (*T. aureum*) (ATCC 34304) revealed not only ARA and EPA but also longer chain PUFAs such as adrenic acid (ADA, 22:4n-6),  $\omega 6$ -docosapentaenoic acid ( $\omega 6$ -DPA, 22:5n-6),  $\omega 3$ -docosapentaenoic acid ( $\omega 3$ -DPA, 22:5n-3), and docosahexaenoic  
10 acid (DHA, 22:6n-3). Thus, in addition to  $\Delta 6$ -,  $\Delta 5$ - and  $\Delta 17$ -desaturases, it was thought that *T. aureum* perhaps contained a  $\Delta 19$ -desaturase which converts ADA to  $\omega 3$ -DPA or  $\omega 6$ -DPA to DHA and/or a  $\Delta 4$ -desaturase which desaturates ADA to  $\omega 6$ -DPA or  $\omega 3$ -DPA to DHA. The goal thus was to attempt to isolate these  
15 predicted desaturase genes from *S. diclina* and *T. aureum*, and eventually to verify the functionality by expression in an alternate host.

To isolate genes encoding functional desaturase enzymes, a cDNA library was constructed for each organism. *Saprolegnia*  
20 *diclina* (ATCC 56851) cultures were grown in potato dextrose media Difco # 336 (Difco Laboratories, Detroit, Michigan) at room temperature for 4 days with constant agitation. The mycelia were harvested by filtration through several layers of cheese cloth, and the cultures crushed in liquid nitrogen using  
25 a mortar and pestle. Total RNA was purified from it using the Qiagen RNeasy Maxi kit (Qiagen, Valencia, CA) as per manufacturer's protocol.

*T. aureum* (ATCC 34304) cells were grown in BY+ Media (Difco #790) at room temperature for 4 days, in the presence of  
30 light, and with constant agitation (250 rpm) to obtain the maximum biomass. These cells were harvested by centrifugation

at 5000 rpm for 10 minutes and rinsed in ice-cold RNase-free water. These cells were then lysed in a French press at 10,000 psi, and the lysed cells directly collected into TE buffered phenol. Proteins from the cell lysate were removed by repeated  
5 phenol:chloroform (1:1 v/v) extraction, followed by a chloroform extraction. The nucleic acids from the aqueous phase were precipitated out at -70°C for 30 minutes using 0.3M (final concentration) sodium acetate (pH 5.6) and one volume of isopropanol. The precipitated nucleic acids were collected by  
10 centrifugation at 15,000rpm for 30 minutes at 4°C, vacuum-dried for 5 minutes and then treated with DNaseI (RNase-free) in 1X DNase buffer (20 mM Tris-Cl, pH 8.0; 5mM MgCl<sub>2</sub>) for 15 minutes at room temperature. The reaction was quenched with 5 mM EDTA (pH 8.0) and the RNA further purified using the Qiagen RNeasy  
15 Maxi kit (Qiagen, Valencia, CA) as per the manufacturer's protocol.

mRNA was isolated from total RNA from each organism using oligo dT cellulose resin. The pBluescript II XR library construction kit (Stratagene, La Jolla, CA) was then used to  
20 synthesize double stranded cDNA which was then directionally cloned (5' *EcoRI*/3' *XhoI*) into pBluescript II SK(+) vector. The *S.diclina* and *T. aureum* libraries contained approximately 2.5 x 10<sup>6</sup> clones each with an average insert size of approximately 700 bp. Genomic DNA from PUFA producing cultures  
25 of *S.diclina* and *T. aureum* was isolated by crushing the culture in liquid nitrogen and purified using Qiagen Genomic DNA Extraction Kit (Qiagen, Valencia, CA).

The approach taken was to design degenerate oligonucleotides (i.e., primers) that represent amino acid  
30 motifs that are conserved in known desaturases. These primers could be used in a PCR reaction to identify a fragment containing the conserved regions in the predicted desaturase

genes from fungi. Since the only fungal desaturases identified are  $\Delta 5$ - and  $\Delta 6$ -desaturase genes from *Mortierella alpina* (Genbank accession numbers AF067650, AB020032, respectively), desaturase sequences from plants as well as animals were taken into consideration during the design of these degenerate primers. Known  $\Delta 5$ - and  $\Delta 6$ -desaturase sequences from the following organisms were used for the design of these degenerate primers: *Mortierella alpina*, *Borago officinalis*, *Helianthus annuus*, *Brassica napus*, *Dictyostelium discoideum*, *Rattus norvegicus*, *Mus musculus*, *Homo sapien*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, and *Ricinus communis*. The degenerate primers used were as follows using the CODEHOP Blockmaker program (<http://blocks.fhcrc.org/codehop.html>):

A. Protein motif 1 (SEQ ID NO:53):

NH<sub>3</sub>- VYDVTEWVKRHPGG -COOH

Primer RO 834 (SEQ ID NO:1):

5'-GTBTAYGAYGTBACCGARTGGGTBAAGCGYCA YCCBGGHGHH-3'

B. Protein Motif 2 (SEQ ID NO:54):

NH<sub>3</sub>- GASANWWKHQHNVHH -COOH

Primer RO835 (Forward) (SEQ ID NO:2):

5'-GGHGCYTCCGCYAACTGGTGAAGCAYCAGCAYAACGTBCAYCAY-3'

Primer RO836 (Reverse) (SEQ ID NO:3 )

5'-RTGRTGVACGTTRTGCTGRTGCTTCCACCAGTTRGCGGARGCDCC-3'

C. Protein Motif 3 (SEQ ID NO:55):

NH<sub>3</sub>- NYQIEHHLFPTM -COOH

Primer RO838 (Reverse) (SEQ ID NO:4)

5'-TTGATRGTCTARCTYGTRGTRGASAARGGVTGGTAC-3'

In addition, two more primers were designed based on the 2nd and 3rd conserved 'Histidine-box' found in known  $\Delta 6$ -desaturases. These were:

5 Primer RO753 (SEQ ID NO:5)  
5'-CATCATCATNGGRAANARRTGRTG-3'  
Primer RO754 (SEQ ID NO:6)  
5'-CTACTACTACTACAYCAYACNTAYACNAAAY-3'

10 The degeneracy code for the oligonucleotide sequences was:  
B=C,G,T; H=A,C,T; S=C,G; R=A,G; V=A,C,G; Y=C,T; D= A+T+C;  
N= A,C,G, or T/U, unknown or other

#### Example 2

15 Isolation of  $\Delta 6$ -Desaturase Nucleotide Sequences from *Saprolegnia diclina* (ATCC 56851)

Total RNA from *Saprolegnia diclina* (ATCC 56851) was isolated using the lithium chloride method (Hoge, et al., Exp. Mycology (1982) 6:225-232). Five  $\mu$ g of the total RNA was reverse transcribed, using the SuperScript Preamplification system (LifeTechnologies, Rockville, MD) and the oligo(dT)<sub>12-18</sub> primer supplied with the kit, to generate the first strand cDNA.

25 To isolate the  $\Delta 6$ -desaturase gene, various permutations and combinations of the above mentioned degenerate oligonucleotides were used in PCR reactions. Of the various primer sets tried, the only primers to give distinct bands were RO834 /RO838. PCR amplification was carried out in a 100  $\mu$ l  
30 volume containing: 2  $\mu$ l of the first strand cDNA template, 20mM Tris-HCl, pH 8.4, 50mM KCl, 1.5mM MgCl<sub>2</sub>, 200  $\mu$ M each deoxyribonucleotide triphosphate and 2 pmole of each primer.



Thermocycling was carried out at two different annealing temperatures, 42°C and 45°C, and these two PCR reactions were combined, resolved on a 1.0% agarose gel, and the band of ~1000bp was gel purified using the QiaQuick Gel Extraction Kit (Qiagen, Valencia, CA). The staggered ends on these fragments were 'filled-in' using T4 DNA polymerase (LifeTechnologies, Rockville, MD) as per manufacturer's specifications, and these DNA fragments were cloned into the PCR-Blunt vector (Invitrogen, Carlsbad, CA). The recombinant plasmids were transformed into TOP10 supercompetent cells (Invitrogen, Carlsbad, CA), and clones were sequenced.

Two clones were thus isolated that showed sequence homology to previously identified  $\Delta 6$ -desaturases. These clones are described as follows:

a. Clone#20-2 was partially sequenced and the deduced amino acid sequence from 702 bp showed 30.2% identity with  $\Delta 6$ -desaturase from *Mortierella alpina* as the highest scoring match in a TfastA search.

b. Clone #30-1 was partially sequenced, and the deduced amino acid sequence of 687 bp showed 48.5% amino acid identity with *Mortierella alpina*'s  $\Delta 6$ -desaturase as the highest scoring match in a TfastA search. These two sequences also overlapped each other indicating they belonged to a single putative  $\Delta 6$ -desaturase from *S. diclina*. This novel  $\Delta 6$ -desaturase sequence was then used to design primers to retrieve the 3'- and the 5'-end of the full-length  $\Delta 6$ -desaturase gene from the cDNA library generated from the mRNA of *S. diclina*.

To isolate the 3'-end, PCR amplification was carried out using plasmid DNA purified from the cDNA library as the template and oligonucleotides RO923 (SEQ ID NO:7) (5'-CGGTGCAGTGGTGGGAAGAACAAGCACAAC-3') and RO899 (SEQ ID NO:8) (5'-AGCGGATAACAATTTTCACACAGGAAACAGC-3'). Oligonucleotide RO923 was designed based on the #20-2 fragment of this putative  $\Delta 6$ -desaturase, and oligonucleotide RO899 corresponded to sequence from the pBluescript II SK(+) vector used for preparation of the cDNA library. Amplification was carried out using 10 pmols of each primer and the Taq PCR Master Mix (Qiagen, Valencia, CA). Samples were denatured initially at 94°C for 3 minutes, followed by 30 cycles of the following: 94°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes. A final extension cycle at 72°C for 10 minutes was carried out before the reaction was terminated. The PCR fragments were resolved on a 0.8% agarose gel and gel purified using the Qiagen Gel Extraction Kit. The staggered end on these fragments were 'filled-in' using T4 DNA polymerase (LifeTechnologies, Rockville, MD) as per manufacturer's specifications, and these DNA fragments were cloned into the PCR-Blunt vector (Invitrogen, Carlsbad, CA). The recombinant plasmids were transformed into TOP10 supercompetent cells (Invitrogen, Carlsbad, CA), and clones were sequenced. Clone sd2-2 contained a 958 bp insert which was identified to contain the 3'-end of the putative  $\Delta 6$ -gene based on sequence homology with known  $\Delta 6$ -desaturases and the presence of the 'TAA' stop codon and Poly A tail.

To isolate the 5'-end of this  $\Delta 6$ -desaturase from *Saprolegnia diclina*, the oligonucleotide RO939 (SEQ ID NO:9) (5'-CGTAGTACTGCTCGAGGAGCTTGAGCGCCG-3') was designed based on the sequence of the #30-1 fragment identified earlier. This oligonucleotide was used in combination with RO898 (SEQ ID NO:10) (5'-CCCAGTCACGACGTTGTAAACGACGGCCAG-3') (designed based

on the sequence of from the pBluescript SK(+) vector) to PCR amplify the 5'-end of the  $\Delta 6$ -desaturase from the cDNA library. In this case, the Advantage-GC cDNA PCR kit (Clontech, Palo Alto, CA) was used to overcome PCR amplification problems that occur with GC rich regions, predicted to be present at the 5'-end of this  $\Delta 6$ -desaturase. PCR thermocycling conditions were as follows: The template was initially denatured at 94°C for 1 minute, followed by 30 cycles of [94°C for 30 seconds, 68°C for 3 minutes], and finally an extension cycle at 68°C for 5 minutes. The PCR products thus obtained were cloned into the PCR-Blunt vector (Invitrogen, Carlsbad, CA) following the same protocol as described above. Clone sd21-2 was thus obtained that contained a 360 bp insert that contained the putative 'ATG' start site of the novel  $\Delta 6$ -desaturase. The deduced amino acid sequence of this fragment, when aligned with known  $\Delta 6$ -desaturases showed 37-45% identity.

This novel  $\Delta 6$ -desaturase gene was isolated in its entirety by PCR amplification using, the *S. diclina* cDNA library, or *S. diclina* genomic DNA as a template, and the following oligonucleotides:

a. RO 951 (SEQ ID NO:11)

(5'- TCAACAGAAATTCATGGTCCAGGGGCAAAAGGCCGAGAAGATCTCG-3')

that contained sequence from the 5' end of clone sd21-2 as well as an *EcoRI* site (underlined) to facilitate cloning into a yeast expression vector

b. RO960 (SEQ ID NO:12)

(5'- ATACGTAAGCTTTTACATGGCGGGAAACTCCTTGAAGAACTCGATCG-3')

that contained sequence from the 3' end of clone sd2-2

including the stop codon as well as a *HindIII* site  
(underlined) for cloning in an expression vector.

PCR amplification was carried out using 200 ng of the cDNA  
5 library plasmid template, 10 pmoles of each primer and the Taq  
PCR Master Mix (Qiagen, Valencia, CA), or 200 ng of genomic  
DNA, 10 pmoles of each primer, and the Advantage-GC cDNA PCR  
kit (Clontech, Palo Alto, CA). Thermocycling conditions were  
as follows: the template was initially denatured at 94°C for 1  
10 minute, followed by 30 cycles of [94°C for 30 seconds, 68°C for  
3 minutes], and finally an extension cycle at 68°C for 5  
minutes. The PCR product thus obtained was digested with  
*EcoRI/HindIII* and cloned into the yeast expression vector  
pYX242 (Invitrogen, Carlsbad, CA) to generate clones pRSP1  
15 (genomic DNA-derived) and pRSP2 (library-derived) which were  
then sequenced and used for expression studies.

The  $\Delta 6$ -desaturase full-length gene insert was 1362 bp (SEQ  
ID NO:13, Figure 2) in length and, beginning with the first  
ATG, contained an open reading frame encoding 453 amino acids.  
20 (The nucleotide sequence encoding the  $\Delta 6$ -desaturase was  
deposited as plasmid pRSP1 with the American Type Culture  
Collection (ATCC), 10801 University Boulevard, Manassas, VA  
20110 under the terms of the Budapest Treaty on January 23,  
2001 and was accorded accession number PTA-2829.) The amino  
25 acid sequence of the full-length gene (SEQ ID NO:14, Figure 3)  
contained regions of homology to  $\Delta 6$ -desaturases from  
*Mortierella alpina*, *Caenorhabditis elegans* and *Borago*  
*officinalis*. It also contained the three conserved 'histidine  
boxes' found in all known membrane-bound desaturases (Okuley,  
30 et al. (1994) *The Plant Cell* 6: 147-158). These were present  
at amino acid positions 171-176, 208-212, and 391-395. As with

other membrane-bound  $\Delta 6$ -desaturases, the third Histidine-box motif (HXXHH) in the *S. diclina*  $\Delta 6$ -desaturase was found to be QXXHH. This sequence also contained a cytochrome b5 domain at the 5'-end. This cytochrome b5 domain is found in a number of  
5 membrane-bound desaturase enzymes, and cytochrome b5 is thought to function as an electron donor in these enzymes. The presence of this domain may be advantageous when expressing the desaturase in heterologous systems for PUFA production. Since the proposed use of this gene is for the reconstruction of the  
10 PUFA biosynthetic pathway in plants, the base composition of this gene may be important. (It is known that some recombinant genes show poor expression because of variations in their base composition as compared to that of the host. The overall G+C content of this gene was 59%, which is close to that of the *M.*  
15 *alpina* desaturases that have been successfully expressed in plants.)

### Example 3

#### Isolation of $\Delta 5$ -Desaturase Nucleotide Sequences from 20 *Saprolegnia diclina* (ATCC 56851)

*Saprolegnia diclina* (ATCC 56851) produces both arachidonic acid (ARA, 20:4 n-6) and eicosapentanoic acid (EPA, 20:5 n-3); thus, it was thought to have, perhaps, a  $\Delta 5$ -desaturase which  
25 can convert dihomo-gamma-linolenic acid (DGLA, 20:3n-6) to arachidonic acid (ARA, 20:4 n-6).

As with the  $\Delta 6$ -desaturase isolation, for the  $\Delta 5$ -desaturase isolation from *S. diclina*, various combinations of the degenerate primers were used in PCR reactions, using first  
30 strand cDNA as the template. The primer combination, RO753 and RO754, generated a distinct band of 588 bp using the following PCR conditions: 2  $\mu$ l of the first strand cDNA template, 20mM



Tris-HCl, pH 8.4, 50mM KCl, 1.5mM MgCl<sub>2</sub>, 200 μM each deoxyribonucleotide triphosphate, 2 pmole of each primer and 1U cDNA polymerase (Clontech, Palo Alto, CA), in a final reaction volume of 50 μl. Thermocycling was carried out as follows: an  
5 initial denaturation at 94°C for 3 minutes, followed by 35 cycles of: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 7 minutes, and the reaction was terminated at 4°C. This fragment thus  
10 generated was cloned (clone # 18-1), sequenced and, when translated, showed 43% amino acid identity with *Mortierella alpina* Δ5-desaturase (Genbank accession # AF067654) and 38.7% identity with *Dictyostelium discoideum* Δ5-desaturase (Genbank accession # AB029311). The second PCR fragment was identified  
15 using Primers R0834 and R0838 in the reaction described in Example 2. This fragment, of approximately 1000 bp in length, was cloned (Clone # 20-8) and the deduced amino acid sequence derived from 775 bp showed 42% identity with Δ5-desaturase from *Dictyostelium discoideum* Δ5-desaturase (Genbank accession #  
20 AB029311). These two sequences, #18-1 and #20-8, overlapped each other indicating they belonged to a single putative Δ5-desaturase from *S. diclina*. These sequences were then used to design primers to retrieve the 3'- and the 5'-end of the novel Δ5-desaturase gene from the cDNA library generated from the  
25 mRNA of *S. diclina*.

To isolate the 3'-end of this putative Δ5-desaturase, PCR amplification was carried out using plasmid DNA purified from the cDNA library, as the template and oligonucleotides R0851 (SEQ ID NO:15) (5'-CCATCAAGACGTACCTTGCGATC-3') and R0899 (SEQ  
30 ID NO:8) (5'- AGCGGATAACAATTTTCACACAGGAAACAGC-3').

Oligonucleotide R0851 was designed based on the #18-1 fragment

of this putative  $\Delta 5$ -desaturase, and oligonucleotide R0899 corresponded to sequence from the pBluescript II SK(+) vector. Amplification was carried out using 200 ng of template plasmid DNA, 10 pmoles of each primer and the Taq PCR Master Mix (Qiagen, Valencia, CA). Samples were denatured initially at 94°C for 3 minutes, followed by 35 cycles of the following: 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minutes. A final extension cycle at 72°C for 7 minutes was carried out before the reaction was terminated. The PCR fragments were cloned into the PCR-Blunt vector (Invitrogen, Carlsbad, CA) as per the protocol described in Example 2. The recombinant plasmids were transformed into TOP10 supercompetent cells (Invitrogen, Carlsbad, CA), and clones were sequenced. Clone sd12-11 contained a 648 bp insert which contained the 3'-end of the putative  $\Delta 5$ -gene based on sequence homology with known  $\Delta 5$ -desaturases and the presence of the 'TAA' stop codon and polyA tail.

The 5'-end of this  $\Delta 5$ -desaturase from *Saprolegnia diclina* was isolated using primers R0941 and R0898. The oligonucleotide R0941 (SEQ ID NO:16) (5'-GCTGAACGGGTGGTACGAGTCGAACGTG-3') was designed based on the sequence of the #20-8 fragment identified earlier. This oligonucleotide was used in combination with R0898 (SEQ ID NO:10) (5'-CCCAGTCACGACGTTGTAAACGACGGCCAG-3') (designed based on the sequence of from the pBluescript II SK(+) vector) in a PCR amplification reaction using the cDNA library plasmid DNA as the template. Here the Advantage-GC cDNA PCR kit (Clontech, Palo Alto, CA) was used as per the manufacturer's protocol, and the thermocycling conditions were as follows: an initial denaturation was carried out at 94°C for 1 minute, followed by 30 cycles of [denaturation at 94°C for 30 seconds, annealing and extension 68°C for 3 minutes], and a final

extension cycle at 68°C for 5 minutes. These PCR products were purified, cloned into the PCR-Blunt vector (Invitrogen, Carlsbad, CA), and sequenced as described above. Clone sd24-1 was identified to contain a 295 bp insert that contained the putative 'ATG' start site of the novel  $\Delta 5$ -desaturase. Analysis of the deduced amino acid sequence of this fragment showed regions of high homology with known  $\Delta 5$ -desaturases and also the presence of a cytochrome b5 domain.

The full-length  $\Delta 5$ -desaturase gene was isolated by PCR amplification using *S. diclina* genomic DNA as a template and the following oligonucleotides:

a. RO 953 (SEQ ID NO:17)

(5'-ACGAGAGAAATTCATGGCCCCGCAGACGGAGCTCCGCCAG

CGC-3') that contained sequence from the 5' end of clone sd24-1 as well as an *EcoRI* site (underlined) to facilitate cloning into a yeast expression vector; and

b. RO956 (SEQ ID NO:18)

(5'-AAAAGACTCGAGTTAGCCCATGTGGATCGTGGCGGCGATGCCC

TGC-3') that contained sequence from the 3' end of clone sd12-11 including the stop codon as well as a *XhoI* site (underlined) for cloning in an expression vector.

Conditions for the PCR amplification of the 'full length' gene were similar to those described for the amplification of the  $\Delta 6$ -desaturase from genomic DNA (Example 2). The PCR product thus obtained was digested with *EcoRI/XhoI* and cloned into the yeast expression vector pYX242 (Invitrogen, Carlsbad, CA).

Clone pRSP3 (genomic DNA-derived) was shown to contain a 1413 bp insert and was used for expression studies.

The 1413 bp full-length gene (SEQ ID NO:19, Figure 4) of the putative  $\Delta 5$ -desaturase from *S.diclina* contained an open reading frame encoding 471 amino acids (SEQ ID NO:20, Figure 5). (The nucleotide sequence encoding the  $\Delta 5$ -desaturase was deposited (as plasmid pRSP3) with the American Type Culture Collection, 10810 University Boulevard, Manassas, VA 20110 under the terms of the Budapest Treaty on January 23, 2001 and was accorded accession number PTA-2928.) This translated protein showed 40.5% overall identity with the *Mortierella alpina*  $\Delta 5$ -desaturase (Genbank accession # AF067654) and 39.5% identity with the *Dictyostelium discoideum*  $\Delta 5$ -desaturase (Genbank accession # AB022097). It also contained the three conserved 'histidine boxes' at amino acid positions 186-190, 223-228, 406-410. Like the  $\Delta 6$ -desaturase, this sequence also contained a cytochrome b5 domain at the 5'-end. The overall G+C content of this gene was 61.5%.

#### Example 4

##### Expression of *S. diclina* Desaturase Genes in Baker's Yeast

20

Clone pRSP2, which consisted of the full length  $\Delta 6$ -desaturase cloned into PYX242 (Invitrogen, Carlsbad, CA), and clone pRSP3, which consisted of the full-length Delta 5-desaturase gene in pYX242, were transformed into competent *Saccharomyces cerevisiae* strain 334. Yeast transformation was carried out using the Alkali-Cation Yeast Transformation Kit (BIO 101, Vista, CA) according to conditions specified by the manufacturer. Transformants were selected for leucine auxotrophy on media lacking leucine (DOB [-Leu]). To detect the specific desaturase activity of these clones, transformants were grown in the presence of 50  $\mu$ M specific fatty acid substrates as listed below:

30

a. Stearic acid (18:0) (conversion to oleic acid would indicate  $\Delta^9$ -desaturase activity)

b. Oleic acid (18:1) (conversion to linoleic acid would indicate  $\Delta^{12}$ -desaturase activity)

5 c. Linoleic acid (18:2 n-6) (conversion to alpha-linolenic acid would indicate  $\Delta^{15}$ -desaturase activity and conversion to gamma-linolenic acid would indicate  $\Delta^6$ -desaturase activity)

10 d. Alpha-linolenic acid (18:3 n-3) (conversion to stearidonic acid would indicate  $\Delta^6$ -desaturase activity)

e. Dihomo-gamma-linolenic acid (20:3 n-6) (conversion to arachidonic acid would indicate  $\Delta^5$ -desaturase activity).

The negative control strain was *S. cerevisiae* 334 containing the unaltered pYX242 vector, and these were grown

15 simultaneously. The cultures were vigorously agitated (250 rpm) and grown for 48 hours at 24°C in the presence of 50  $\mu$ M (final concentration) of the various substrates. The cells

were pelleted and vortexed in methanol; chloroform was added along with tritridecanoin (as an internal standard). These

20 mixtures were incubated for at least an hour at room

temperature or at 4°C overnight. The chloroform layer was

extracted and filtered through a Whatman filter with 1 gm anhydrous sodium sulfate to remove particulates and residual

water. The organic solvents were evaporated at 40°C under a

25 stream of nitrogen. The extracted lipids were then derivitized to fatty acid methyl esters (FAME) for gas chromatography analysis (GC) by adding 2 ml of 0.5 N potassium hydroxide in methanol to a closed tube. The samples were heated to 95°C-100°C for 30 minutes and cooled to room temperature.

30 Approximately 2 ml of 14% borontrifluoride in methanol were added and the heating repeated. After the extracted lipid



mixture cooled, 2 ml of water and 1 ml of hexane were added to extract the FAME for analysis by GC. The percent conversion was calculated by dividing the product produced by the sum of (the product produced + the substrate added) and then  
5 multiplying by 100.

Table 1 represents the enzyme activity of the genes isolated based on the percent conversion of substrate added. The pRSP1 clone that contained the  $\Delta 6$ -desaturase gene from *S. diclina* converted 28% of the 18:2n-6 substrate to 18:3n-3, as  
10 well as 37% of the 18:3n-3 substrate to 18:4n-3. This confirms that the gene encodes a  $\Delta 6$ -desaturase. There was no background (non-specific conversion of substrate) in this case. (All tables referred to herein are presented after the Abstract of the Disclosure.)

15 The pRSP3 clone that contained the  $\Delta 5$ -desaturase gene from *S. diclina* was capable of converting 27% of the added 20:3n-6 substrate to 20:4n-6, indicating that the enzyme it encodes is a  $\Delta 5$ -desaturase. In this case too, there was no background substrate conversion detected. This data indicates that  
20 desaturases with different substrate specificity can be expressed in a heterologous system and can also be used to produce polyunsaturated fatty acids.

Table 2 represents fatty acids of interest as a percentage of the total lipid extracted from *S. cerevisiae* 334 with the  
25 indicated plasmid. No glucose was present in the growth media. Affinity gas chromatography was used to separate the respective lipids. GC/MS was employed to identify the products. From this table, it is apparent that exogenously added substrates, when added in the free form was taken up by the recombinant  
30 yeast and the incorporated into their membranes. In the yeast clone containing the  $\Delta 6$ -desaturase gene (pRSP1), GLA ( $\gamma$ -18:3)

was identified as a novel PUFA when LA (18:2) was added as the substrate, and arachidonic acid was detected in yeast containing the  $\Delta 5$ -desaturase gene (pRSP3) when DGLA (20:3) was added as a substrate.

5

#### Example 5

##### Co-Expression of *S. diclina* Desaturases with Elongases

The plasmid pRSP1 ( $\Delta 6$ ) and pRSP3 ( $\Delta 5$ ) were individually co-transformed with pRAE73-A3, a clone that contains the Human Elongase gene (SEQ ID NO:21) in the yeast expression vector pYES2, into yeast as described in Example 4. This elongase gene catalyzes some of the elongation steps in the PUFA pathway. Co-transformants were selected on minimal media lacking leucine and uracil (DOB[-Leu-Ura]).

Table 3 shows that when 50  $\mu$ M of the substrate LA (18:2 n-6) was added, that the  $\Delta 6$ -desaturase converted this substrate to GLA (18:3 n-6) and the elongase was able to add two carbons to GLA to produce DGLA (20:3 n-6). The percent conversion of the substrate to the final product by these co-transformed enzymes is 26.4%, with no background observed from the negative control. Similarly, the co-transformed enzymes can act on ALA (18:3n-3) to finally form (20:4n-3) with a percentage conversion of 34.39%. Thus, *S. diclina*  $\Delta 6$ -desaturase was able to produce a product in a heterologous expression system that could be further utilized by another heterologous enzyme from the PUFA biosynthetic pathway to produce the expected PUFA.

Table 4 shows results of the pRSP3( $\Delta 5$ )/Human Elongase co-transformation experiment. In this case, substrate GLA (18:3n-6) was converted to DGLA (20:3n-6) by human elongase and this was further converted to ARA (20:4n-6) by the action of *S.*

*diclina*  $\Delta 5$ -desaturase. The percent conversion of the substrate to the final product by these co-transformed enzymes is 38.6%, with no background observed from the negative control.

The other substrate tested in this case was STA (18:4  
5 n-3) which was eventually converted to EPA (20:5n-3) by the concerted action of the two enzymes. Similar results were observed when the pRSP1 and pRSP3 were cotransformed with an elongase gene derived from *M. alpina* (pRPB2) (SEQ ID NO:22), and both genes were shown to be functional in the presence of  
10 each other (see Table 3 and Table 4).

#### Example 6

##### Isolation of $\Delta 5$ -Desaturase Nucleotide Sequences from *Thraustochytrium aureum* (ATCC 34303)

15 To isolate putative desaturase genes, total RNA was isolated as described in Example 2. Approximately 5  $\mu$ g was reverse transcribed using the SuperScript Preamplification system (LifeTechnologies, Rockville, MD) as shown in Example 2  
20 to produce first strand cDNA. Using the degenerate primers RO834 (SEQ ID NO:1) and 838 (SEQ ID NO:4) designed with the block maker program in a 50  $\mu$ l reaction, the following components were combined: 2  $\mu$ l of the first strand cDNA template, 20mM Tris-HCl, pH 8.4, 50mM KCl, 1.5mM MgCl<sub>2</sub>, 200  $\mu$ M  
25 each deoxyribonucleotide triphosphate, 2 pmole final concentration of each primer and cDNA polymerase (Clonetech, Palo Alto, CA). Thermocycling was carried out as follows: an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C  
30 for 30 seconds and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 7 minutes. Two faint bands of approximately 1000 bp were separated on a 1% agarose

gel, excised, and purified with the QiaQuick Gel Extraction Kit (Qiagen, Valencia, CA). The ends were filled in with T4 DNA polymerase and the blunt-end fragments cloned into PCR Blunt as described in Example 2. Sequencing of the obtained clones  
5 identified the partial sequence of 680 bp from clone 30-9 whose translation of 226 amino acids had 31.5% identity with  $\Delta 6$ -desaturase from adult zebrafish (Genbank accession number AW281238). A similar degree of amino acid (29.6%- 28.7%) homology was found with human  $\Delta 6$ -desaturase (Genbank accession  
10 number AF126799), *Physcomitrella patens* (moss)  $\Delta 6$ -desaturase (Genbank accession number AJ222980), *Brassica napus* (canola)  $\Delta 8$ -sphingolipid desaturase (Genbank accession number AJ224160), and human  $\Delta 5$ -desaturase (ATCC accession number 203557, Genbank accession number AF199596). Since there was a reasonable  
15 degree of amino acid homology to known desaturases, a full-length gene encoding a potential desaturase was sought to determine its activity when expressed in yeast.

To isolate the 3' end of the gene, 10 pmol of primer RO936 (SEQ ID NO:23) (5'-GTCGGGCAAGGCGGAAAAGTACCTCAAGAG-3')  
20 and vector primer RO899 (SEQ ID NO:8) were combined in a reaction with 100 ng of purified plasmid from the *T. aureum* cDNA library in reaction volume of 100  $\mu$ l in Taq PCR Master Mix (Qiagen, Valencia, CA). Thermocycling conditions were as follows: an initial melt at 94°C for 3 minutes followed by 30  
25 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes. This was followed by an extension step of 10 minutes at 72°C. Several bands, including the predicted size of 1.2 kb, were separated on a 1 % agarose gel and purified as stated earlier. Also as described earlier, the ends of the fragments  
30 were blunt ended,  
cloned into PCR Blunt and sequenced. Fragment #70-2 of

approximately 1.2 kb was sequenced and contained an open reading frame and a stop codon, which overlapped fragment 30-9.

To isolate the 5' end of the gene, RO937 (SEQ ID NO:24) (5'-AAACCTGTAGACAATGTGGAGGGGCGTGGG-3') and RO 899 (SEQ ID NO:8)

5 were used in a 50 µl PCR reaction with Advantage-GC cDNA PCR kit (Clontech, Palo Alto, CA), as per the manufacturer's protocol, with 100 ng of purified plasmid DNA from the library and 10 pmol of each primer. The thermocycling conditions were as follows: An

10 initial denaturation was carried out at 94°C for 1 minute, followed by 30 cycles of [denaturation at 94°C for 30 seconds, annealing and extension 68°C for 3 minutes], and a final extension cycle at 68°C for 5 minutes. A band of approximately 500bp, in the range of the expected size, was gel  
15 purified, blunt ended and cloned into PCR Blunt as previously described. Clone 95-2 contained an open reading frame with a start codon. This fragment also overlapped with clone 30-9, indicating that they were indeed pieces of the same gene.

To isolate the full-length gene, primers were designed  
20 with restriction sites 5' and 3' (underlined) with *EcoRI* and *XhoI*, respectively, as follows: 5' primer RO972 (SEQ ID NO:25) (5'-ATACTTGAATTCATGGGACGCGGCGGCGAAGGTCAGGTGAAC-3'), 3' primer RO949 (SEQ ID NO: 26) (5'-CTTATACTCGAGCTAAGCGG CCTTGGCCGCCGCTGGCC-3') and 3' primer RO950 (SEQ ID NO:27) (5'-  
25 CTTATACTCGAGTAAATGGCTCGCGAGGCGAAGCGAGTGGC-3'). Two primers were used for the 3' end of the gene in the initial isolation attempt since the primer RO949, containing the stop codon had 66% GC content, while the alternate primer RO950, which was outside the stop codon, had only a 56% GC content. A 50 µl PCR  
30 reaction with RO972/RO949 and RO972/950 was performed with Advantage-GC cDNA PCR kit (Clontech, Palo Alto, CA) under



identical conditions noted in the preceding paragraph. Only the primer set R0972/950 produced a band of approximately 1.6 kb. Use of genomic DNA as a template (under identical conditions with 100 ng of target) also produced a similar-sized band. Fragments were separated on an agarose gel, gel purified, blunt-ended and cloned into PCR Blunt as previously described. Fragments were evaluated by sequencing, and a number of clones were cut with *EcoRI/XhoI* to excise the full length gene, ligated to pYX242 *EcoRI/XhoI* which had been treated with shrimp alkaline phosphatase (Roche, Indianapolis, IN) with the Rapid ligation kit (Roche, Indianapolis, IN). Clone 99-3, designated pRTA4, contained the full length gene of 1317 bp (SEQ ID NO:28, Figure 6) and an open reading frame of 439 aa (SEQ IN NO: 29, Figure 7). (The nucleotide sequence encoding the  $\Delta 5$ -desaturase was deposited with the ATCC, 10801 University Boulevard, Manassas, VA 20110 under the terms of the Budapest Treaty on January 23, 2001 and was accorded accession number PTA-2927.) This gene contained three histidine boxes at amino acid numbers 171-175, 208-212, and 376-380. The 5'-end of the gene, when translated, also shows homology to cytochrome b5.

#### Example 7

##### Expression of *T. aureum* Desaturase Gene in Baker's Yeast

The clone pRTA4 containing the full-length gene was transformed into the yeast host *S. cerevisiae* 334 and plated on selective media as described in Example 4. The cultures were grown at 24°C for 48 hours in minimal media lacking leucine with 50  $\mu$ M of exogenous free fatty acid added as a substrate as shown in Table 5. The only conversion of a substrate was DGLA (20:3n-6) to ARA (20:4n-6). The conversion of 23.7% of the

added DGLA indicates that this gene encodes for a  $\Delta 5$ -desaturase.

Table 6 shows some of the fatty acids as a percentage of the lipid extracted from the yeast host. For  $\Delta 5$ -desaturase activity, there was no background (detection of ARA observed in the negative control containing the yeast expression plasmid, PYX242.)

#### Example 8

##### 10 Co-Expression of *T. aureum* Desaturase Gene with Elongases

The plasmid pRTA4 was co-transformed with an additional enzyme in the PUFA pathway, pRAE73-A3 which contains the human elongase gene in the yeast expression vector pYES2 as described in Example 4, and co-transformants were selected on minimal media lacking leucine and uracil.

Table 7 shows that when 100  $\mu\text{M}$  of the substrate DGLA was added, that the  $\Delta 5$ -desaturase actively produced ARA, to which the elongase was able to add two carbons to produce ADA. The percent conversion of *T. aureum*  $\Delta 5$ -desaturase, which consists of both ARA and ADA (products), was 16.7%, with no background observed from the negative control.

In view of the above results, *T. aureum*  $\Delta 5$ -desaturase is able to produce a product in a heterologous expression system that can be used by an additional heterologous enzyme in the PUFA biosynthetic pathway to produce the expected PUFA.

#### Example 9

##### 30 Isolation of $\Delta 5$ -desaturase Nucleotide Sequences from *Thraustochytrium aureum* BICC7091 (T7091)

A partial desaturase candidate was isolated using the degenerate primer combination of RO834/RO838, listed in Example 1. The genomic DNA was prepared from Thraustochytrium aureum BICC7091 (Biocon India Ltd., Bangalore, India) using the DNeasy plant maxi kit (Qiagen, Valencia, CA). The T7091 gDNA was amplified with primers RO834 (5' -GTB TAY GAY GTB ACC GAR TGG GTB AAG CGY CAY CCB GGH GGH- 3') (SEQ ID NO:1) and RO838 (5' -CAT GGT VGG RAA SAG RTG RTG YTC RAT CTG RTA GTT- 3') (SEQ ID NO:36). PCR amplification was carried out in a 100  $\mu$ l volume containing: 5  $\mu$ l of isolated T7091 gDNA, 0.2 M dNTP mix, 50 pM each primer, 10  $\mu$ l of 10X buffer and 1.0 U of cDNA Polymerase. Thermocycler conditions in Perkin Elmer 9600 were as follows: 94°C for 3 min, then 35 cycles of 94 °C for 30 sec., 60 °C for 30 sec., and 72°C for 1 min. PCR was followed by an additional extension at 72°C for 7 minutes. The PCR amplified mixture was run on a 1.0% agarose gel, and amplified fragments of approximately 1.2 Kb and 1.4 Kb were gel purified using the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA). The staggered ends on these fragments were filled-in using T4 DNA Polymerase (LifeTechnologies, Rockville, MD), the isolated fragments were cloned into the pCR-Blunt vector (Invitrogen, Co., Carlsbad, CA), and the recombinant plasmids were transformed into TOP10 supercompetent cells (Invitrogen, Carlsbad, CA).

Twenty-four clones were prepared and sequenced using ABI 373A DNA Sequencer (Applied Biosystems, Foster City, CA). The translated sequences were used as queries to search the GenEmbl database (Genetics Computer Group (GCG) (Madison, WI)), using the tFastA algorithm (a Pearson and Lipman search for similarity between a protein query sequence and any group of nucleotide sequences). A gene fragment, T7091B2, was further pursued because it had identity with known desaturases.

T7091B2 had 26.8% identity in 362 amino acids with Human  $\Delta 5$ -desaturase (GenBank accession number AF226273).

To isolate the 3' and 5'-ends, new primers were designed based on the T7091B2 internal sequence. The T7091 cDNA

5 library, which contains approximately  $2 \times 10^7$  clones with an average insert size of 1 Kb, was PCR amplified using the new primers and a vector primer. The primers that produced more 5' and 3' sequences with the vector primers RO898 (5' -CCC AGT CAC GAC GTT GTA AAA CGA CGG CCA G- 3') (SEQ ID NO:10) and RO899 (5' 10 -AGC GGA TAA CAA TTT CAC ACA GGA AAC AGC- 3') (SEQ ID NO:8) were RO1065 (5' -CGA CAA GAG GAA GAG TGT CCA AAT C- 3') (SEQ ID NO:37) and RO1064 (5' -CGC CTT CAA GAG TTT TTG TAC GGA ATT GGG- 3') (SEQ ID NO:38), respectively, for clone T7091B2. Two new primers were designed based on the T7091B2 5' and 3' sequences:

15

- a. RO1097 (5' -CTT GTA CCA TGG GTC GCG GAG CAC AGG GAG- 3) (SEQ ID NO:39), which has an added *NcoI* restriction site (underlined)
- b. RO1098 (5' -TGA AGC TTA CTC GCT CTT GGC AGC TTG GCC- 20 3') (SEQ ID NO:40), which has an added *HindIII* restriction site (underlined)

This novel  $\Delta 5$ -desaturase gene was isolated in its entirety by PCR amplification, using the *T. aureum* 7091 gDNA. PCR was 25 carried out in a 50  $\mu$ l volume containing:

1  $\mu$ l of isolated T7091 gDNA, 0.2  $\mu$ M dNTP mix, 50 pM each primer, 5  $\mu$ l of 10 X buffer, 1.5  $\mu$ l of 50 mM  $MgSO_4$ , and 0.5 U of *Taq* DNA Polymerase. Thermocycler conditions in Perkin Elmer 9600 were as follows: 94°C for 3 min, then 30 cycles of 95 °C 30 for 45 sec., 55 °C for 30 sec., and 68°C for 2 min. The PCR amplified mixture was run on a gel, an amplified fragment of

approximately 1.3 Kb was gel purified, and the isolated fragment was cloned into the pYX242(NcoI/EcoRV) vector. Two clones, designated as pRAT-2a and 2c, were prepared and sequenced. (Plasmid pRAT-2c was deposited with the American  
5 Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209 on January \_\_\_, 2002 under the terms of the Budapest Treaty and was accorded ATCC deposit number \_\_\_\_\_.) The sequences were different by four amino acids (Figure 12), and the translated sequence had 67.4% identity in 436 amino acids  
10 with T. aureum (ATCC34303)  $\Delta 5$ -desaturase (see Example 6).

#### Example 10

##### Isolation of $\Delta 6$ -desaturase Nucleotide Sequences from *Thraustochytrium aureum* BICC7091 (T7091)

15

Three hundred and seventy-three templates from the T7091 cDNA library were sequenced to determine the viability of the library. The translated sequences were used as queries to search the GenEmbl database using the tFastA algorithm. Clone  
20 602187281R1 (or clone 281 for short) had identity with several known desaturases. The 5' and 3' ends of the EST clone were sequenced in order to design primers for amplification. Primers RO1107 (5' -TTT AAC CAT GGG CCG CGG CGG CGA GAA AAG-3') (SEQ ID NO:41), which has an added NcoI restriction site  
25 (underlined), and RO1108 (5' -GGG AAG AAG CTT TCT ACT GCG CCT TGG CTT TCT TTG- 3') (SEQ ID NO:42), which has an added *HindIII* restriction site (underlined), were used to PCR amplify the T7091 gDNA.

PCR was carried out in a 50  $\mu$ l volume with *Taq* DNA  
30 Polymerase as above. The PCR amplified mixture was run on a gel, an amplified fragment of approximately 1.3 Kb was gel purified, and the isolated fragment was cloned into the



pYX242 (*NcoI*/*EcoRV*) vector. Two clones, designated as pRAT-1a and 1b, were prepared and sequenced. The sequences were different by one amino acid (Figure 13), and the translated sequence had 25% identity in 430 amino acids with the Human  $\Delta 5$ -desaturase. (Plasmid pRAT-1a was deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209 on January \_\_\_, 2002 under the terms of the Budapest Treaty and was accorded ATCC deposit number \_\_\_\_.)

#### Example 11

##### Expression of *T. aureum* 7091 Desaturase Genes in Baker's Yeast

Clones pRAT-2a and pRAT-2c, which consisted of the full-length  $\Delta 5$ -desaturase cloned into pYX242 (Invitrogen, Carlsbad, CA), and clones pRAT-1a and pRAT-1b, which consisted of the full-length  $\Delta 6$ -desaturase gene in pYX242, were transformed into competent *Saccharomyces cerevisiae* 334. Yeast transformation was carried out using the Alkali-Cation Yeast Transformation Kit (BIO 101, Vista, CA). Transformants were selected for leucine auxotrophy on media lacking leucine (DOB[-leu]). Because the translated sequence of pRAT-1 cDNA did not have a strong identity with any particular known desaturase, several fatty acid substrates were tested to determine the activity of the expressed enzyme. To detect the specific desaturase activity of pRAT-1a and pRAT-1b clones, transformants were grown in the presence of 100  $\mu$ M specific fatty acid substrates as listed below:

- a. Linoleic acid (LA, 18:2n-6) (conversion to  $\alpha$ -linolenic acid would indicate  $\Delta 15$ -desaturase activity and conversion to  $\gamma$ -linolenic acid would indicate  $\Delta 6$ -desaturase activity)

- b.  $\alpha$ -linolenic acid (ALA, 18:3n-3) (conversion to stearidonic acid would indicate  $\Delta$ 6-desaturase activity)
- c.  $\omega$ 6-eicosadienoic acid (EDA, 20:2n-6) (conversion to dihomono- $\gamma$ -linolenic acid would indicate  $\Delta$ 8-desaturase activity)
- d. dihomono- $\gamma$ -linolenic acid (DGLA, 20:3n-6) (conversion to arachidonic acid would indicate  $\Delta$ 5-desaturase activity)

The substrate for pRAT-2 clones was 100  $\mu$ M of DGLA. S. cerevisiae 334 containing the unaltered pYX242 vector was used as a negative control. Both pRAT-1a and pRAT-2c were also co-transformed into *S. cerevisiae* 334 with pRAE-73-A3 (i.e, a vector containing the human elongase enzyme which converts 18C fatty acids to 20C fatty acids, SEQ ID NO:21) (see Example 5). The substrates for pRAT-1a/pRAE-73-A3 clones were LA and ALA, and the substrates for pRAT-2c/pRAE-73-A3 clones were LA, ALA, and GLA. The cultures were grown for 48 hours at 24°C, in selective media, in the presence of a particular substrate. Fatty acid analyses were performed as outlined in Example 4.

Table 8 includes an example of the production of AA by strain 334 (pRAT-2c) vs. that by the control strain 334 (pYX242). The amount of AA produced was 22.98%, vs. no detectable amount in the control strain. Strain 334 (pRAT-2a) also had no detectable amount of AA produced (data not shown). The four amino acid difference between the two clones (Figure 12, see underlined) rendered the enzyme expressed from strain 334 (pRAT-2a) to be inactive. The  $\Delta$ 5-desaturase activity from clone pRAT-2c was further detected when co-expressed with pRAE-73-A3 in the presence of GLA.

Table 9 is the fatty acid analysis results of the strains 334 (pRAT-1a) and 334 (pYX242) expressed in the presence of substrates LA, ALA, EDA, and DGLA. The  $\Delta 6$ -desaturation of LA will produce GLA, the  $\Delta 8$ -desaturation of EDA will produce DGLA, and the  $\Delta 5$ -desaturation of DGLA will produce AA. All three activities were detected from both strains containing the T7091 gene vs. the control strain. Based on the conversion rates, this enzyme is an active  $\Delta 6$ -desaturase, which can behave like a  $\Delta 5$ - or  $\Delta 8$ -desaturase, given the proper substrates. However, the conversion rates of these substrates to their respective desaturated fatty acids are low, in comparison to the substrates for a  $\Delta 6$ -desaturation. The single amino acid difference between the two clones (Figure 13, see underlined) had no affect on the enzymatic activity (data not shown). The percent conversions are shown in the lower box of Table 9. The  $\Delta 6$ -desaturase activity was further detected when pRAT-1a was co-expressed with pRAE-73-A3 in the presence of LA or ALA. The percent conversions were not calculated for the co-expression experiment, since it is difficult to determine whether the production of the new fatty acids is due to the activity of the desaturase, or elongase, or the combination of the two enzymes.

Two different methods of isolating a novel gene were explored in order to identify desaturase genes from T. aureum 7091. These methods led to the isolation of the T7091  $\Delta 5$ -desaturase and  $\Delta 6$ -desaturase genes. In the presence of these genes and the appropriate substrates, S. cerevisiae produced desaturated fatty acids at a much higher level than the control strain. Co-transformation of the constructs containing the T7091B2 gene (pRAT-2c) and the Human elongase gene (pRAE-73-A3) in yeast resulted in the conversion of the substrate GLA to AA.

This experiment confirmed that the enzyme expressed from pRAT-2c must desaturate the DGLA (produced by the elongase) to AA. Co-transformation of the constructs containing the ``281'' gene (pRAT-1a) and the Human elongase gene (pRAE-73-A3) in yeast  
 5 resulted in the conversion of the substrate LA to DGLA. This experiment confirmed that the enzyme expressed from pRAT-1a must desaturate LA to GLA.

### Example 13

#### 10      Isolation of $\Delta 5$ -desaturase Nucleotide Sequence from *Isochrysis galbana* 1323

A partial desaturase candidate was isolated using the degenerate primer combination of RO834/RO838, listed in Example  
 15 1. The genomic DNA was prepared from *Isochrysis galbana* CCMP1323 (Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP), West Boothbay Harbor, MA) using the DNeasy plant maxi kit (Qiagen, Valencia, CA). The *I. galbana* gDNA was amplified with primers RO834 (5' -GTB TAY GAY  
 20 GTB ACC GAR TGG GTB AAG CGY CAY CCB GGH GGH- 3') (SEQ ID NO:1) and RO838 (5' -CAT GGT VGG RAA SAG RTG RTG YTC RAT CTG RTA GTT- 3') (SEQ ID NO:10). PCR was carried out in a 50  $\mu$ l volume containing: 1  $\mu$ l of isolated *I. galbana* gDNA, 0.2 mM dNTP mix, 50 pM each primer, 5  $\mu$ l of 10 X buffer, 1.5  $\mu$ l of 50 mM MgSO<sub>4</sub>,  
 25 and 0.5 U of Taq DNA Polymerase. Thermocycler conditions in Perkin Elmer 9600 were as follows: 94°C for 3 min, then 30 cycles of 95 °C for 45 sec., 55 °C for 30 sec., and 68°C for 2 min. The PCR amplified mixture was run on a 1.0% agarose gel, and an amplified fragment of approximately 1.1 Kb was gel  
 30 purified using the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA). The staggered ends of the fragment were filled-in using T4 DNA Polymerase (LifeTechnologies, Rockville, MD),

the isolated fragment was cloned into the pCR-Blunt vector (Invitrogen, Co., Carlsbad, CA), and the recombinant plasmids were transformed into TOP10 supercompetent cells (Invitrogen, Carlsbad, CA).

5 Six clones were prepared and sequenced using ABI 373A DNA Sequencer (Applied Biosystems, Foster City, CA). All of the sequences were the same. The translated sequence of the isolated fragment had 47.5% identity in 335 amino acids with with T. aureum (ATCC 34303)  $\Delta$ 5-desaturase in clone pRTA4  
10 (Example 6), and 45.3% identity in 278 amino acids with T. aureum BICC7091  $\Delta$ 5-desaturase in clone pRAT-2c (Example 9).

To isolate the 5' and 3'-ends, new primers were designed based on the internal sequence of the isolated I. galbana fragment. For the 5 prime end of the gene RO1235 (5'- CGA AGT  
15 TGG TGA AGA TGT AGG TGC CG-3') (SEQ ID NO:43) was used, while RO1232 (5'-GAG CGA CGC GTA CAA CAA CTT TCA CGT-3') (SEQ ID NO:44) was used for the 3 prime end of the gene. Approximately 1.4  $\mu$ g of total RNA was used, according to the manufacturer's direction, with the Rapid amplification of cDNA  
20 ends or RACE with the GeneRacer™ kit (Invitrogen, Carlsbad, CA) and Superscript II™ enzyme (Invitrogen, Carlsbad, CA) for reverse transcription to produce cDNA target. For the initial amplification of the ends, the following thermocycling protocol was used in a Perkin Elmer 9600: initial melt at 94°C for 2  
25 minutes; followed by 5 cycles of 94°C for 30 seconds and 72°C for 3 minutes; 10 cycles of 94°C 30 seconds, 70°C for 30 seconds, and 72°C for 3 minutes; and 20 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 3 minutes; followed by an extension of 72°C for 10 minutes. This first PCR  
30 reaction was performed with 10 pMol of RO1235 or RO1232 and GeneRacer™ 5 prime primer (5'- CGA CTG GAG CAC GAG GAC ACT GA-



3') (SEQ ID NO:45), or GeneRacer™ 3 prime primer (5'- GCT GTC AAC GAT ACG CTA CGT AAC G-3') (SEQ ID NO:46), respectively, with 1 l of Thermozyme™ (Invitrogen, Carlsbad, CA) and 1 ~~µ~~l of cDNA in a final volume of 50 ~~µ~~l, according to the manufacturer's directions.

A nested reaction was performed with 2 ~~µ~~l of the initial reaction, 10 pmol of nested primer RO1234 (5'-AGC TCC AGG TGA TTG TGC ACG CGC AG-3') (SEQ ID NO:47) or RO1233 (5'- GAC TTT GAG AAG CTG CGC CTC GAG CTG-3') (SEQ ID NO:48) and 30 pmol the GeneRacer™ nested 5 prime primer (5'- GGA CAC TGA CAT GGA CTG AAG GAG TA-3') (SEQ ID NO:49) and GeneRacer™ nested 3 prime primer (5'- CGC TAC GTA ACG GCA TGA CAG TG -3') (SEQ ID NO:50) respectively, and Platinum Taq™ PCRx (Clonetech, Palo Alto, CA) using MgSO<sub>4</sub> according to the manufacturer's protocol. The thermocycling parameter was as follows in a Perkin Elmer 9600: initial melt at 94°C for 2 minutes; followed by 5 cycles of 94°C for 30 seconds and 72°C for 2 minutes; 5 cycles of 94°C 30 seconds, 70°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 65°C for 30 seconds and 68°C for 2 minutes; followed by an extension of 68°C for 10 minutes. Agarose gel analysis of the PCR products showed a band around 800 base pairs for the 5 prime reactions and approximately a 1.2 kilobase band for 3 prime reaction. Subsequent cloning into pCR Blunt (Invitrogen, Carlsbad, CA), transformation into Top10 competent cells (Invitrogen, Carlsbad, CA), and sequencing, revealed an open reading frame with both a start and stop codons.

Primers RO1309 (5'- ATG ATG GAA TTC ATG GTG GCA GGC AAA TCA GGC GC-3') (SEQ ID NO:51) and RO1310 (5'- AAT AAT GTC GAC CTA GTG CGT GTG CTC GTG GTA GG-3') (SEQ ID NO:52) with restrictions sites added for cloning (see underlined *EcoRI*, and *SalI*, respectively) were used to isolate a full length gene.

As shown above, 10 pmol of primers R01309 and 1310 were used with Platinum Taq™ PCRx (Clonetech, Palo Alto, CA) using MgSO<sub>4</sub> according to the manufacturer's protocol, with 2 μl of the cDNA as target. The thermocycling parameters were as follows:

5 initial melt at 94°C for 2 minutes; followed by 5 cycles of 94°C for 30 seconds and 72°C for 2 minutes; 5 cycles of 94°C 30 seconds, 70°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 65°C for 30 seconds and 68°C for 2 minutes; followed by an extension of 68°C for 10 minutes. The single product of the  
10 reaction was gel purified using the QiaQuick gel purification kit (Qiagen, Valencia, CA), cut with *EcoRI* and *Sall*, ligated to pYX242 *EcoRI*/*XhoI* linearized DNA with the Rapid ligation kit (Roche, Indianapolis, IN), and designated pRIG-1. The clone pRIG-1 contained a full length gene of 1329 bp (SEQ ID NO:34;  
15 Figure 14) and an open reading frame of 442 amino acid (SEQ ID NO:35; Figure 15). (Plasmid pRIG-1 was deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209 on January \_\_\_, 2002 under the terms of the Budapest Treaty and was accorded ATCC deposit number \_\_\_\_.)

20

### Example 13

#### Expression of *I. galbana* Desaturase Gene in Baker's Yeast

25

The clone pRIG-1 containing the full-length gene was transformed into the yeast host *S. cerevisiae* 334 and plated on selective media as described in Example 4. The cultures were grown at 24°C for 48 hours in minimal media lacking leucine,  
30 with 50 μM of exogenous free fatty acid added as a substrate as shown in Table 10. The conversion of substrates was ETA (20:4n-3) to EPA (20:5n-3) and DGLA (20:3n-6) to AA (20:4n-6).

The 45.4% conversion to ARA and 59.75% conversion to EPA indicate that this gene encodes for a  $\Delta 5$ -desaturase. Table 10 shows some of the fatty acids as a percentage of the lipid extracted from the yeast host. For  $\Delta 5$ -desaturase activity, there was little or no background (detection of ARA or EPA observed in the negative control containing the yeast expression plasmid, pYX242.)

#### Example 14

##### Co-Expression of *I. galbana* Desaturase Gene with Elongases

The plasmid pRIG-1 could be co-transformed with an additional enzyme in the PUFA pathway, such as pRAE-73-A3 which contains the human elongase gene in the yeast expression vector pYES2 as described in Example 4, and co-transformants selected on minimal media lacking leucine and uracil. Substrates such as DGLA or ETA could be added so that the  $\Delta 5$ -desaturase would actively produced ARA or EPA, to which the elongase is able to add two carbons to produce ADA or  $\Delta 3$ -DPA. Therefore, *I. galbana*  $\Delta 5$ -desaturase could produce a product in a heterologous expression system that can be used by an additional heterologous enzyme in the PUFA biosynthetic pathway, to produce the expected PUFA.

##### Nutritional Compositions

The PUFAs described in the Detailed Description may be utilized in various nutritional supplements, infant formulations, nutritional substitutes and other

nutritional solutions.

## I. INFANT FORMULATIONS

### A. Isomil® Soy Formula with Iron:

5

Usage: As a beverage for infants, children and adults with an allergy or sensitivity to cows milk. A feeding for patients with disorders for which lactose should be avoided: lactase deficiency, lactose intolerance and galactosemia.

10

#### Features:

-Soy protein isolate to avoid symptoms of cow's-milk-protein allergy or sensitivity.

15

-Lactose-free formulation to avoid lactose-associated diarrhea.

-Low osmolality (240 mOs/kg water) to reduce risk of osmotic diarrhea.

20

-Dual carbohydrates (corn syrup and sucrose) designed to enhance carbohydrate absorption and reduce the risk of exceeding the absorptive capacity of the damaged gut.

-1.8 mg of Iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.

-Recommended levels of vitamins and minerals.

25

-Vegetable oils to provide recommended levels of essential fatty acids.

-Milk-white color, milk-like consistency and pleasant aroma.

30

Ingredients: (Pareve) 85% water, 4.9% corn syrup, 2.6% sugar (sucrose), 2.1 % soy oil, 1.9% soy protein isolate, 1.4% coconut oil, 0.15% calcium citrate, 0.11 % calcium phosphate tribasic, potassium citrate, potassium phosphate

monobasic, potassium chloride, mono- and diglycerides, soy lecithin, carrageenan, ascorbic acid, L-methionine, magnesium chloride, potassium phosphate dibasic, sodium chloride, choline chloride, taurine, ferrous sulfate, m-  
5 inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone,  
10 biotin, sodium selenite, vitamin D3 and cyanocobalamin.

B. Isomil® DF Soy Formula For Diarrhea:

15 Usage: As a short-term feeding for the dietary management of diarrhea in infants and toddlers.

Features:

- 20 -First infant formula to contain added dietary fiber from soy fiber specifically for diarrhea management.
- Clinically shown to reduce the duration of loose, watery stools during mild to severe diarrhea in infants.
- Nutritionally complete to meet the nutritional needs of the infant.
- 25 -Soy protein isolate with added L-methionine meets or exceeds an infant's requirement for all essential amino acids.
- Lactose-free formulation to avoid lactose-associated diarrhea.
- 30 -Low osmolality (240 mOsm/kg water) to reduce the risk of osmotic diarrhea.



-Dual carbohydrates (corn syrup and sucrose) designed to enhance carbohydrate absorption and reduce the risk of exceeding the absorptive capacity of the damaged gut.

-Meets or exceeds the vitamin and mineral levels

5 recommended by the Committee on Nutrition of the American Academy of Pediatrics and required by the Infant Formula Act.

-1.8 mg of iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.

10 -Vegetable oils to provide recommended levels of essential fatty acids.

Ingredients: (Pareve) 86% water, 4.8% corn syrup, 2.5% sugar (sucrose), 2.1% soy oil, 2.0% soy protein isolate, 15 1.4% coconut oil, 0.77% soy fiber, 0.12% calcium citrate, 0.11% calcium phosphate tribasic, 0.10% potassium citrate, potassium chloride, potassium phosphate monobasic, mono and diglycerides, soy lecithin, carrageenan, magnesium chloride, ascorbic acid, L-methionine, potassium phosphate 20 dibasic, sodium chloride, choline chloride, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine 25 hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D3 and cyanocobalamin.

C. Isomil® SF Sucrose-Free Soy Formula With Iron:

30

Usage: As a beverage for infants, children and adults with an allergy or sensitivity to cow's-milk protein or an

intolerance to sucrose. A feeding for patients with disorders for which lactose and sucrose should be avoided.

Features:

- 5        -Soy protein isolate to avoid symptoms of cow's-milk-protein allergy or sensitivity.
- Lactose-free formulation to avoid lactose-associated diarrhea (carbohydrate source is Polycose® Glucose Polymers).
- 10       -Sucrose free for the patient who cannot tolerate sucrose.
- Low osmolality (180 mOsm/kg water) to reduce risk of osmotic diarrhea.
- 1.8 mg of iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.
- 15       -Recommended levels of vitamins and minerals.
- Vegetable oils to provide recommended levels of essential fatty acids.
- Milk-white color, milk-like consistency and pleasant aroma.

20

Ingredients: (Pareve) 75% water, 11.8% hydrolyzed cornstarch, 4.1% soy oil, 4.1 % soy protein isolate, 2.8% coconut oil, 1.0% modified cornstarch, 0.38% calcium phosphate tribasic, 0.17% potassium citrate, 0.13%

25

potassium chloride, mono- and diglycerides, soy lecithin, magnesium chloride, ascorbic acid, L-methionine, calcium carbonate, sodium chloride, choline chloride, carrageenan, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium

30

pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D3 and cyanocobalamin.

D. Isomil® 20 Soy Formula With Iron Ready To Feed,  
20 Cal/fl oz.:

Usage: When a soy feeding is desired.

5

10

15

20

Ingredients: (Pareve) 85% water, 4.9% corn syrup, 2.6%  
sugar(sucrose), 2.1 % soy oil, 1.9% soy protein isolate,  
1.4% coconut oil, 0.15% calcium citrate, 0. 11% calcium  
phosphate tribasic, potassium citrate, potassium phosphate  
monobasic, potassium chloride, mono- and diglycerides, soy  
lecithin, carrageenan, ascorbic acid, L-methionine,  
magnesium chloride, potassium phosphate dibasic, sodium  
chloride, choline chloride, taurine, ferrous sulfate, m-  
inositol, alpha-tocopheryl acetate, zinc sulfate, L-  
carnitine, niacinamide, calcium pantothenate, cupric  
sulfate, vitamin A palmitate, thiamine chloride  
hydrochloride, riboflavin, pyridoxine hydrochloride, folic  
acid, manganese sulfate, potassium iodide, phylloquinone,  
biotin, sodium selenite, vitamin D3 and cyanocobalamin.

25

E. Similac® Infant Formula:

Usage: When an infant formula is needed: if the decision  
is made to discontinue breastfeeding before age 1 year, if  
a supplement to breastfeeding is needed or as a routine  
feeding if breastfeeding is not adopted.

30

Features:

-Protein of appropriate quality and quantity for good  
growth; heat-denatured, which reduces the risk of milk-  
associated enteric blood loss.  
-Fat from a blend of vegetable oils (doubly homogenized),  
providing essential linoleic acid that is easily absorbed.

-Carbohydrate as lactose in proportion similar to that of human milk.

-Low renal solute load to minimize stress on developing organs.

5 -Powder, Concentrated Liquid and Ready To Feed forms.

Ingredients: (-D) Water, nonfat milk, lactose, soy oil, coconut oil, mono- and diglycerides, soy lecithin, ascorbic acid, carrageenan, choline chloride, taurine, m-  
10 inositol, alpha-tocopheryl acetate, zinc sulfate, niacinamide, ferrous sulfate, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, phylloquinone, biotin, sodium  
15 selenite, vitamin D3 and cyanocobalamin.

F.Similac® NeoCare Premature Infant Formula With Iron:

Usage: For premature infants' special nutritional needs  
20 after hospital discharge. Similac NeoCare is a nutritionally complete formula developed to provide premature infants with extra calories, protein, vitamins and minerals needed to promote catch-up growth and support development.

25

Features:

-Reduces the need for caloric and vitamin supplementation. More calories (22 Cal/fl oz) than standard term formulas (20 Cal/fl oz).

30

-Highly absorbed fat blend, with medium-chain triglycerides (MCToil) to help meet the special digestive needs of premature infants.

-Higher levels of protein, vitamins and minerals per 100 calories to extend the nutritional support initiated in-hospital.

-More calcium and phosphorus for improved bone mineralization.

Ingredients: -D Corn syrup solids, nonfat milk, lactose, whey protein concentrate, soy oil, high-oleic safflower oil, fractionated coconut oil (medium chain triglycerides), coconut oil, potassium citrate, calcium phosphate tribasic, calcium carbonate, ascorbic acid, magnesium chloride, potassium chloride, sodium chloride, taurine, ferrous sulfate, m-inositol, choline chloride, ascorbyl palmitate, L-carnitine, alpha-tocopheryl acetate, zinc sulfate, niacinamide, mixed tocopherols, sodium citrate, calcium pantothenate, cupric sulfate, thiamine chloride hydrochloride, vitamin A palmitate, beta carotene, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, phylloquinone, biotin, sodium selenite, vitamin D3 and cyanocobalamin.

G. Similac Natural Care Low-Iron Human Milk Fortifier Ready To Use, 24 Cal/fl oz.:

Usage: Designed to be mixed with human milk or to be fed alternatively with human milk to low-birth-weight infants.

Ingredients: -D Water, nonfat milk, hydrolyzed cornstarch, lactose, fractionated coconut oil (medium-chain triglycerides), whey protein concentrate, soy oil, coconut oil, calcium phosphate tribasic, potassium citrate, magnesium chloride, sodium citrate, ascorbic acid, calcium carbonate, mono and diglycerides, soy lecithin, carrageenan, choline chloride, m-inositol, taurine,



niacinamide, L-carnitine, alpha tocopheryl acetate, zinc sulfate, potassium chloride, calcium pantothenate, ferrous sulfate, cupric sulfate, riboflavin, vitamin A palmitate, thiamine chloride hydrochloride, pyridoxine hydrochloride, biotin, folic acid, manganese sulfate, phylloquinone, vitamin D3, sodium selenite and cyanocobalamin.

Various PUFAs of this invention can be substituted and/or added to the infant formulae described above and to other infant formulae known to those in the art.

## II. NUTRITIONAL FORMULATIONS

### A. ENSURE®

Usage: ENSURE is a low-residue liquid food designed primarily as an oral nutritional supplement to be used with or between meals or, in appropriate amounts, as a meal replacement. ENSURE is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets. Although it is primarily an oral supplement, it can be fed by tube.

#### Patient Conditions:

- For patients on modified diets
- For elderly patients at nutrition risk
- For patients with involuntary weight loss
- For patients recovering from illness or surgery
- For patients who need a low-residue diet

Ingredients: -D Water, Sugar (Sucrose), Maltodextrin (Corn), Calcium and Sodium Caseinates, High-Oleic Safflower Oil, Soy Protein Isolate, Soy Oil, Canola Oil, Potassium Citrate, Calcium Phosphate Tribasic, Sodium Citrate, Magnesium Chloride, Magnesium Phosphate Dibasic, Artificial Flavor, Sodium Chloride, Soy Lecithin, Choline

Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate,  
Ferrous Sulfate, Alpha-Tocopheryl Acetate, Gellan Gum,  
Niacinamide,  
Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate,  
5 Vitamin A Palmitate, Thiamine Chloride Hydrochloride,  
Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Sodium  
Molybdate, Chromium Chloride, Biotin, Potassium Iodide,  
Sodium Selenate.

10 B.ENSURE® BARS:

Usage: ENSURE BARS are complete, balanced nutrition for  
supplemental use between or with meals. They provide a  
delicious, nutrient-rich alternative to other snacks.

15 ENSURE BARS contain <1 g lactose/bar, and Chocolate Fudge  
Brownie flavor is gluten-free. (Honey Graham Crunch flavor  
contains gluten.)

Patient Conditions:

20 -For patients who need extra calories, protein, vitamins  
and minerals.

-Especially useful for people who do not take in enough  
calories and nutrients.

-For people who have the ability to chew and swallow

25 -Not to be used by anyone with a peanut allergy or any  
type of allergy to nuts.

Ingredients: Honey Graham Crunch -- High-Fructose Corn  
Syrup, Soy Protein Isolate, Brown Sugar, Honey,

Maltodextrin (Corn), Crisp Rice (Milled Rice,

30 Sugar [Sucrose], Salt [Sodium Chloride] and Malt), Oat  
Bran, Partially Hydrogenated Cottonseed and Soy Oils, Soy  
Polysaccharide, Glycerine, Whey Protein Concentrate,  
Polydextrose, Fructose, Calcium Caseinate, Cocoa Powder,  
Artificial Flavors, Canola Oil, High-Oleic Safflower Oil,

Nonfat Dry Milk, Whey Powder, Soy Lecithin and Corn Oil.  
Manufactured in a facility that processes nuts.

Vitamins and Minerals: Calcium Phosphate Tribasic,  
Potassium Phosphate Dibasic, Magnesium Oxide, Salt (Sodium  
Chloride), Potassium Chloride, Ascorbic Acid, Ferric  
Orthophosphate, Alpha-Tocopheryl Acetate, Niacinamide,  
Zinc Oxide, Calcium Pantothenate, Copper Gluconate,  
Manganese Sulfate, Riboflavin, Beta Carotene, Pyridoxine  
Hydrochloride, Thiamine Mononitrate, Folic Acid, Biotin,  
Chromium Chloride, Potassium Iodide, Sodium Selenate,  
Sodium Molybdate, Phylloquinone, Vitamin D3 and  
Cyanocobalamin.

Protein: Honey Graham Crunch - The protein source is a  
blend of soy protein isolate and milk proteins.

Soy protein isolate	74%
Milk proteins	26%

Fat: Honey Graham Crunch - The fat source is a blend of  
partially hydrogenated cottonseed and soybean, canola,  
high oleic safflower, oils, and soy lecithin.

Partially hydrogenated cottonseed and soybean oil	76%
Canola oil	8%
High-oleic safflower oil	8%
Corn oil	4%
Soy lecithin	4%

Carbohydrate: Honey Graham Crunch - The carbohydrate  
source is a combination of high-fructose corn syrup, brown

sugar, maltodextrin, honey, crisp rice, glycerine, soy polysaccharide, and oat bran.

	High-fructose corn syrup	24%
5	Brown sugar	21%
	Maltodextrin	12%
	Honey	11%
	Crisp rice	9%
	Glycerine	9%
10	Soy Polysaccharide	7%
	Oat bran	7%

#### C. ENSURE® HIGH PROTEIN:

15 Usage: ENSURE HIGH PROTEIN is a concentrated, high-protein liquid food designed for people who require additional calories, protein, vitamins, and minerals in their diets. It can be used as an oral nutritional supplement with or between meals or, in appropriate amounts, as a meal  
20 replacement. ENSURE HIGH PROTEIN is lactose- and gluten-free, and is suitable for use by people recovering from general surgery or hip fractures and by patients at risk for pressure ulcers.

#### 25 Patient Conditions:

-For patients who require additional calories, protein, vitamins, and minerals, such as patients recovering from general surgery or hip fractures, patients at risk for pressure ulcers, and patients on low-cholesterol diets.

30

#### Features:

-Low in saturated fat  
-Contains 6 g of total fat and < 5 mg of cholesterol per serving

- Rich, creamy taste
- Excellent source of protein, calcium, and other essential vitamins and minerals
- For low-cholesterol diets
- 5 -Lactose-free, easily digested

#### Ingredients:

10 Vanilla Supreme: -D Water, Sugar (Sucrose), Maltodextrin (Corn), Calcium and Sodium Caseinates, High-Oleic Safflower Oil, Soy Protein Isolate, Soy Oil, Canola Oil, Potassium Citrate, Calcium Phosphate Tribasic, Sodium Citrate, Magnesium Chloride, Magnesium Phosphate Dibasic, Artificial Flavor, Sodium Chloride, Soy Lecithin, Choline

15 Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Gellan Gum, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride,

20 Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Sodium Molybdate, Chromium Chloride, Biotin, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D3 and Cyanocobalamin.

#### 25 Protein:

The protein source is a blend of two high-biologic-value proteins: casein and soy.

30	Sodium and calcium caseinates	85%
	Soy protein isolate	15%

#### Fat:

The fat source is a blend of three oils: high-oleic safflower, canola, and soy.



High-oleic safflower oil	40%
Canola oil	30%
Soy oil	30%

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The level of fat in ENSURE HIGH PROTEIN meets American Heart Association (AHA) guidelines. The 6 grams of fat in ENSURE HIGH PROTEIN represent 24% of the total calories, with 2.6% of the fat being from saturated fatty acids and 7.9% from polyunsaturated fatty acids. These values are within the AHA guidelines of < 30% of total calories from fat, < 10% of the calories from saturated fatty acids, and < 10% of total calories from polyunsaturated fatty acids.

15

#### Carbohydrate:

20

ENSURE HIGH PROTEIN contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla supreme, chocolate royal, wild berry, and banana), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

#### Vanilla and other nonchocolate flavors:

25

Sucrose	60%
Maltodextrin	40%

#### Chocolate:

30

Sucrose	70%
Maltodextrin	30%

D. ENSURE® LIGHT

Usage: ENSURE LIGHT is a low-fat liquid food designed for use as an oral nutritional supplement with or between meals. ENSURE LIGHT is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets.

#### Patient Conditions:

-For normal-weight or overweight patients who need extra nutrition in a supplement that contains 50% less fat and 20% fewer calories than ENSURE.

-For healthy adults who do not eat right and need extra nutrition.

#### Features:

-Low in fat and saturated fat

-Contains 3 g of total fat per serving and < 5 mg cholesterol

-Rich, creamy taste

-Excellent source of calcium and other essential vitamins and minerals

-For low-cholesterol diets

-Lactose-free, easily digested

#### Ingredients:

French Vanilla: -D Water, Maltodextrin (Corn), Sugar (Sucrose), Calcium Caseinate, High-Oleic Safflower Oil, Canola Oil, Magnesium Chloride, Sodium Citrate, Potassium Citrate, Potassium Phosphate Dibasic, Magnesium Phosphate Dibasic, Natural and Artificial Flavor, Calcium Phosphate Tribasic, Cellulose Gel, Choline Chloride, Soy Lecithin, Carrageenan, Salt (Sodium Chloride), Ascorbic Acid, Cellulose Gum, Ferrous Sulfate, Alpha-Tocopheryl Acetate,

Zinc Sulfate, Niacinamide, Manganese Sulfate, Calcium  
Pantothenate, Cupric Sulfate, Thiamine Chloride  
Hydrochloride, Vitamin A Palmitate, Pyridoxine  
Hydrochloride, Riboflavin, Chromium Chloride, Folic Acid,  
5 Sodium Molybdate, Biotin, Potassium Iodide, Sodium  
Selenate, Phylloquinone, Vitamin D3 and Cyanocobalamin.

Protein:

The protein source is calcium caseinate.

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Calcium caseinate	100%
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Fat:

15

The fat source is a blend of two oils: high-oleic  
safflower and canola.

High-oleic safflower oil	70%
Canola oil	30%

20

The level of fat in ENSURE LIGHT meets American Heart  
Association (AHA) guidelines. The 3 grams of fat in ENSURE  
LIGHT represent 13.5% of the total calories, with 1.4% of  
the fat being from saturated fatty acids and 2.6%  
25 from polyunsaturated fatty acids. These values are within  
the AHA guidelines of < 30% of total calories from fat, <  
10% of the, calories from saturated fatty acids, and < 10%  
of total calories from polyunsaturated fatty acids.

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Carbohydrate:

ENSURE LIGHT contains a combination of maltodextrin and  
sucrose. The chocolate flavor contains corn syrup as  
well. The mild sweetness and flavor variety (French  
vanilla, chocolate supreme, strawberry swirl), plus VARI-

FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

5

Vanilla and other nonchocolate flavors:

Sucrose	51%
Maltodextrin	49%

10

Chocolate:

Sucrose	47.0%
Corn Syrup	26.5%
Maltodextrin	26.5%

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Vitamins and Minerals:

An 8-fl-oz serving of ENSURE LIGHT provides at least 25% of the RDIs for 24 key vitamins and minerals.

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Caffeine:

Chocolate flavor contains 2.1 mg caffeine/8 fl oz.

E.ENSURE PLUS®

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Usage: ENSURE PLUS is a high-calorie, low-residue liquid food for use when extra calories and nutrients, but a normal concentration of protein, are needed. It is designed primarily as an oral nutritional supplement to be used

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with or between meals or, in appropriate amounts, as a meal replacement. ENSURE PLUS is lactose- and gluten-free. Although it is primarily an oral nutritional supplement, it can be fed by tube.

Patient Conditions:

-For patients who require extra calories and nutrients,  
but a normal concentration of protein, in a limited  
volume.

5 -For patients who need to gain or maintain healthy weight.

Features:

-Rich, creamy taste

-Good source of essential vitamins and minerals

10

Ingredients:

Vanilla: -D Water, Corn Syrup, Maltodextrin (Corn), Corn  
Oil, Sodium and Calcium Caseinates, Sugar (Sucrose), Soy  
15 Protein Isolate, Magnesium Chloride, Potassium Citrate,  
Calcium Phosphate Tribasic, Soy Lecithin, Natural and  
Artificial Flavor, Sodium Citrate, Potassium Chloride,  
Choline Chloride, Ascorbic Acid, Carrageenan, Zinc  
Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate,  
20 Niacinamide, Calcium Pantothenate, Manganese Sulfate,  
Cupric  
Sulfate, Thiamine Chloride Hydrochloride, Pyridoxine  
Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic  
Acid, Biotin, Chromium Chloride, Sodium Molybdate;  
25 Potassium Iodide, Sodium Selenite, Phylloquinone,  
Cyanocobalamin and Vitamin D3.

Protein:

30 The protein source is a blend of two high-biologic-  
value proteins: casein and soy.

Sodium and calcium caseinates	84%
Soy protein isolate	16%



## Fat:

The fat source is corn oil.

Corn oil 100%

## Carbohydrate:

ENSURE PLUS contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla, chocolate, strawberry, coffee, butter pecan, and eggnog), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla, strawberry, butter pecan, and coffee flavors:

Corn Syrup	39%
Maltodextrin	38%
Sucrose	23%

Chocolate and eggnog flavors:

Corn Syrup	36%
Maltodextrin	34%
Sucrose	30%

## Vitamins and Minerals:

An 8-fl-oz serving of ENSURE PLUS provides at least 15% of the RDIs for 25 key Vitamins and minerals.

## Caffeine:

Chocolate flavor contains 3.1 mg Caffeine/8 fl oz. Coffee flavor contains a trace amount of caffeine.

F. ENSURE PLUS® HN

Usage: ENSURE PLUS HN is a nutritionally complete high-calorie, high-nitrogen liquid food designed for people with higher calorie and protein needs or limited volume tolerance. It may be used for oral supplementation or for total nutritional support by tube. ENSURE PLUS HN is lactose- and gluten-free.

Patient Conditions:

- For patients with increased calorie and protein needs, such as following surgery or injury.
- For patients with limited volume tolerance and early satiety.

Features:

- For supplemental or total nutrition
- For oral or tube feeding
- 1.5 CaV/mL,
- High nitrogen
- Calorically dense

Ingredients:

Vanilla: -D Water, Maltodextrin (Corn), Sodium and Calcium Caseinates, Corn Oil, Sugar (Sucrose), Soy Protein Isolate, Magnesium Chloride, Potassium Citrate, Calcium Phosphate Tribasic, Soy Lecithin, Natural and Artificial Flavor, Sodium Citrate, Choline Chloride, Ascorbic Acid, Taurine, L-Carnitine, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Niacinamide, Carrageenan, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic Acid, Biotin, Chromium Chloride, Sodium Molybdate, Potassium Iodide,

Sodium Selenite, Phylloquinone, Cyanocobalamin and Vitamin D3.

G.ENSURE® POWDER:

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Usage: ENSURE POWDER (reconstituted with water) is a low-residue

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liquid food designed primarily as an oral nutritional supplement to be used with or between meals. ENSURE POWDER is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets.

Patient Conditions:

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- For patients on modified diets
- For elderly patients at nutrition risk
- For patients recovering from illness/surgery
- For patients who need a low-residue diet

Features:

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- Convenient, easy to mix
- Low in saturated fat
- Contains 9 g of total fat and < 5 mg of cholesterol per serving
- High in vitamins and minerals
- For low-cholesterol diets
- Lactose-free, easily digested

25

30

Ingredients: -D Corn Syrup, Maltodextrin (Corn), Sugar (Sucrose), Corn Oil, Sodium and Calcium Caseinates, Soy Protein Isolate, Artificial Flavor, Potassium Citrate, Magnesium Chloride, Sodium Citrate, Calcium Phosphate Tribasic, Potassium Chloride, Soy Lecithin, Ascorbic Acid, Choline Chloride, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Niacinamide, Calcium Pantothenate,

Manganese Sulfate, Thiamine Chloride Hydrochloride, Cupric Sulfate, Pyridoxine Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic Acid, Biotin, Sodium Molybdate, Chromium Chloride, Potassium Iodide, Sodium Selenate,  
5 Phylloquinone, Vitamin D3 and Cyanocobalamin.

Protein:

The protein source is a blend of two high-biologic-value proteins: casein and soy.

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Sodium and calcium caseinates	84%
Soy protein isolate	16%

Fat:

15

The fat source is corn oil.

Corn oil	100%
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Carbohydrate:

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ENSURE POWDER contains a combination of corn syrup, maltodextrin, and sucrose. The mild sweetness of ENSURE POWDER, plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, helps to prevent flavor fatigue and aid in patient compliance.

25

Vanilla:

Corn Syrup	35%
Maltodextrin	35%
30 Sucrose	30%

H. ENSURE® PUDDING

Usage: ENSURE PUDDING is a nutrient-dense supplement providing balanced nutrition in a nonliquid form to be used with or between meals. It is appropriate for consistency-modified diets (e.g., soft, pureed, or full liquid) or for people with swallowing impairments. ENSURE PUDDING is gluten-free.

Patient Conditions:

- For patients on consistency-modified diets (e.g., soft, pureed, or full liquid)
- For patients with swallowing impairments

Features:

- Rich and creamy, good taste
- Good source of essential vitamins and minerals
- Convenient-needs no refrigeration
- Gluten-free

Nutrient Profile per 5 oz: Calories 250, Protein 10.9%, Total Fat 34.9%, Carbohydrate 54.2%

Ingredients:

Vanilla: -D Nonfat Milk, Water, Sugar (Sucrose), Partially Hydrogenated Soybean Oil, Modified Food Starch, Magnesium Sulfate, Sodium Stearoyl Lactylate, Sodium Phosphate Dibasic, Artificial Flavor, Ascorbic Acid, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Choline Chloride, Niacinamide, Manganese Sulfate, Calcium Pantothenate, FD&C Yellow #5, Potassium Citrate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, FD&C Yellow #6, Folic Acid, Biotin, Phylloquinone, Vitamin D3 and Cyanocobalamin.



## Protein:

The protein source is nonfat milk.

5	Nonfat milk	100%
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## Fat:

The fat source is hydrogenated soybean oil.

10	Hydrogenated soybean oil	100%
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## Carbohydrate:

15	ENSURE PUDDING contains a combination of sucrose and modified food starch. The mild sweetness and flavor variety (vanilla, chocolate, butterscotch, and tapioca) help prevent flavor fatigue. The product contains 9.2 grams of lactose per serving.
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20	Vanilla and other nonchocolate flavors:
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	Sucrose	56%
	Lactose	27%
	Modified food starch	17%

25	
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## Chocolate:

	Sucrose	58%
	Lactose	26%
30	Modified food starch	16%

## I. ENSURE® WITH FIBER:

Usage: ENSURE WITH FIBER is a fiber-containing, nutritionally complete liquid food designed for people who

can benefit from increased dietary fiber and nutrients. ENSURE WITH FIBER is suitable for people who do not require a low-residue diet. It can be fed orally or by tube, and can be used as a nutritional supplement to a regular diet or, in appropriate amounts, as a meal replacement. ENSURE WITH FIBER is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets.

10 Patient Conditions:

-For patients who can benefit from increased dietary fiber and nutrients

Features:

- 15 -New advanced formula-low in saturated fat, higher in vitamins and minerals
- Contains 6 g of total fat and < 5 mg of cholesterol per serving
- Rich, creamy taste
- 20 -Good source of fiber
- Excellent source of essential vitamins and minerals
- For low-cholesterol diets
- Lactose- and gluten-free

25 Ingredients:

Vanilla: -D Water; Maltodextrin (Corn), Sugar (Sucrose), Sodium and Calcium Caseinates, Oat Fiber, High-Oleic Safflower Oil, Canola Oil, Soy Protein Isolate, Corn Oil, Soy Fiber, Calcium Phosphate Tribasic, Magnesium Chloride, Potassium Citrate, Cellulose Gel, Soy Lecithin, Potassium Phosphate Dibasic, Sodium Citrate, Natural and Artificial Flavors, Choline Chloride, Magnesium Phosphate, Ascorbic Acid, Cellulose Gum, Potassium Chloride, Carrageenan, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Zinc Sulfate,

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Niacinamide, Manganese Sulfate, Calcium Pantothenate,  
Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride  
Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Folic  
Acid, Chromium Chloride, Biotin, Sodium Molybdate,  
5 Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin  
D3 and Cyanocobalamin.

Protein:

10 The protein source is a blend of two high-biologic-  
value proteins-casein and soy.

Sodium and calcium caseinates	80%
Soy protein isolate	20%

15 Fat:

The fat source is a blend of three oils: high-oleic  
safflower, canola, and corn.

20 High-oleic safflower oil	40%
Canola oil	40%
Corn oil	20%

25 The level of fat in ENSURE WITH FIBER meets American Heart  
Association (AHA) guidelines. The 6 grams of fat in ENSURE  
WITH FIBER represent 22% of the total calories, with 2.01  
% of the fat being from saturated fatty acids and 6.7%  
from polyunsaturated fatty acids. These values are within  
the AHA guidelines of  $\leq 30\%$  of total calories from fat, <  
30 10% of the calories from saturated fatty acids, and  $\leq 10\%$   
of total calories from polyunsaturated fatty acids.

Carbohydrate:

ENSURE WITH FIBER contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla, chocolate, and butter pecan), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla and other nonchocolate flavors:

10	Maltodextrin	66%
	Sucrose	25%
	Oat Fiber	7%
	Soy Fiber	2%

15 Chocolate:

	Maltodextrin	55%
	Sucrose	36%
	Oat Fiber	7%
20	Soy Fiber	2%

Fiber:

The fiber blend used in ENSURE WITH FIBER consists of oat fiber and soy polysaccharide. This blend results in approximately 4 grams of total dietary fiber per 8-fl. oz can. The ratio of insoluble to soluble fiber is 95:5.

The various nutritional supplements described above and known to others of skill in the art can be substituted and/or supplemented with the PUFAs produced in accordance with the present invention.

## J.Oxepa™ Nutritional Product

Oxepa is a low-carbohydrate, calorically dense, enteral nutritional product designed for the dietary management of patients with or at risk for ARDS. It has a unique combination of ingredients, including a patented oil blend containing eicosapentaenoic acid (EPA from fish oil),  $\gamma$ -linolenic acid (GLA from borage oil), and elevated antioxidant levels.

## Caloric Distribution:

Caloric density is high at 1.5 Cal/mL (355 Cal/8 fl oz), to minimize the volume required to meet energy needs. The distribution of Calories in Oxepa is shown in Table A.

Table A. Caloric Distribution of Oxepa

	per 8 fl oz.	per liter	% of Cal
Calories	355	1,500	---
Fat (g)	22.2	93.7	55.2
Carbohydrate (g)		25	105.528.1
Protein (g)	14.8	62.5	16.7
Water (g)	186	785	---

## Fat:

-Oxepa contains 22.2 g of fat per 8-fl oz serving (93.7 g/L).

-The fat source is an oil blend of 31.8% canola oil, 25% medium-chain triglycerides (MCTs), 20% borage oil, 20% fish oil, and 3.2 % soy lecithin. The typical fatty acid profile of Oxepa is shown in Table B.

-Oxepa provides a balanced amount of polyunsaturated, monounsaturated, and saturated fatty acids, as shown in Table VI.



-Medium-chain triglycerides (MCTs) -- 25% of the fat blend  
-- aid gastric emptying because they are absorbed by the  
intestinal tract without emulsification by bile acids.

5

The various fatty acid components of Oxepa<sup>TM</sup> nutritional  
product can be substituted and/or supplemented with the  
PUFAs produced in accordance with this invention.

Table B. Typical Fatty Acid Profile

		% Total	g/8 fl oz*	g/L*
5	Fatty Acids			
	Caproic (6:0)	0.2	0.04	0.18
	Caprylic (8:0)	14.69	3.1	13.07
10	Capric (10:0)	11.06	2.33	9.87
	Palmitic (16:0)	5.59	1.18	4.98
	Palmitoleic	1.82	0.38	1.62
	Stearic	1.94	0.39	1.64
	Oleic	24.44	5.16	21.75
15	Linoleic	16.28	3.44	14.49
	$\alpha$ -Linolenic	3.47	0.73	3.09
	$\gamma$ -Linolenic	4.82	1.02	4.29
	Eicosapentaenoic	5.11	1.08	4.55
	n-3-Docosapent-	0.55	0.12	0.49
20	aenoic			
	Docosahexaenoic	2.27	0.48	2.02
	Others	7.55	1.52	6.72
25	Fatty acids equal approximately 95% of total fat.			

Table C. Fat Profile of Oxepa.

% of total calories from fat	55.2
Polyunsaturated fatty acids	31.44 g/L
Monounsaturated fatty acids	25.53 g/L
5 Saturated fatty acids	32.38 g/L
n-6 to n-3 ratio	1.75:1
Cholesterol	9.49 mg/8 fl oz
	40.1 mg/L

## Carbohydrate:

10 -The carbohydrate content is 25.0 g per 8-fl-oz serving (105.5 g/L).

-The carbohydrate sources are 45% maltodextrin (a complex carbohydrate) and 55% sucrose (a simple sugar), both of which are readily digested and absorbed.

15 -The high-fat and low-carbohydrate content of Oxepa is designed to minimize carbon dioxide (CO<sub>2</sub>) production. High CO<sub>2</sub> levels can complicate weaning in ventilator-dependent patients. The low level of carbohydrate also may be useful for those patients who have developed stress-induced

20 hyperglycemia.

-Oxepa is lactose-free.

Dietary carbohydrate, the amino acids from protein, and the glycerol moiety of fats can be converted to glucose

25 within the body. Throughout this process, the carbohydrate requirements of glucose-dependent tissues (such as the central nervous system and red blood cells) are met. However, a diet free of carbohydrates can lead to ketosis, excessive catabolism of tissue protein, and loss of fluid

30 and electrolytes. These effects can be prevented by daily ingestion of 50 to 100 g of digestible carbohydrate, if caloric intake is adequate. The carbohydrate level in Oxepa is also sufficient to minimize gluconeogenesis, if energy needs are being met.

Protein:

-Oxepa contains 14.8 g of protein per 8-fl-oz serving  
(62.5 g/L).

5 -The total calorie/nitrogen ratio (150:1) meets the need  
of stressed patients.

-Oxepa provides enough protein to promote anabolism and  
the maintenance of lean body mass without precipitating  
respiratory problems. High protein intakes are a concern  
10 in patients with respiratory insufficiency. Although  
protein has little effect on CO<sub>2</sub> production, a high  
protein diet will increase ventilatory drive.

-The protein sources of Oxepa are 86.8% sodium caseinate  
and 13.2% calcium caseinate.

15 -The amino acid profile of the protein system in Oxepa  
meets or surpasses the standard for high quality protein  
set by the National Academy of Sciences.

\* Oxepa is gluten-free.

Table 1

*Saprolegnia diclina* (ATCC 56851) Desaturase Expression in Baker's Yeast

CLONE	TYPE OF ENZYME ACTIVITY	% CONVERSION OF SUBSTRATE	
pRSP1 ( <i>S. diclina</i> $\Delta 6$ desaturase)	$\Delta 9$	0	(18:0 to 18:1n-9)*
	$\Delta 12$	0	(18:1 to 18:2n-6)
	$\Delta 15$	0	(18:2n-6 to 18:3n-3)
	$\Delta 6$	28	(18:2n-6 to 18:3n-6)
	$\Delta 6$	37	(18:3n-3 to 18:4n-3)
	$\Delta 5$	0	(20:3n-6 to 20:4n-6)
pRSP3 ( <i>S. diclina</i> $\Delta 5$ desaturase)	$\Delta 9$	0	(18:0 to 18:1n-9)
	$\Delta 12$	0	(18:1 to 18:2n-6)
	$\Delta 15$	0	(18:2n-6 to 18:3n-3)
	$\Delta 6$	0	(18:2n-6 to 18:3n-6)
	$\Delta 6$	0	(18:3n-3 to 18:4n-3)
	$\Delta 5$	27	(20:3n-6 to 20:4n-6)

\*above endogenous  $\Delta 9$  activity



Table 2  
Fatty Acid as a Percentage of Total Lipid Extracted from Yeast

Clone	18:1* Present	18:2 (n-3) Produced	18:2 (n-6) Incorporated	18:3 (n-3) Produced	18:3 (n-6) Produced	18:3 (n-3) Incorporated	18:4 (n-3) Produced	20:3(n-6) Incorporated	20:4 (n-6) Produced
pYX242 (control)	15.07	0	11.14	0	0	11.35	0	11.55	0
pRSP1 (Δ6)	14.41	0	6.31	0	2.44	7.95	4.63	13.70	0
pRSP3 (Δ5)	15.34	0.08	10.72	0	0	10.43	0	20.69	7.74

50 μM substrate added  
\*18:1 is an endogenous fatty acid in yeast

- Key:
- 18:1 = Oleic acid
  - 18:2 (n-6) = Linoleic acid
  - 18:3 (n-3) = α-Linolenic acid
  - 18:3 (n-6) = γ-Linolenic acid
  - 18:4 (n-3) = Stearidonic acid
  - 20:3 (n-6) = Dihomo-γ-linolenic acid
  - 20:4 (n-6) = Arachidonic acid

Table 3

Fatty Acid as a Percentage of Total Lipid Extracted from Yeast

Clone	Plasmid in yeast (enzyme)	18:2 (n-6) Incorporated	18:3 (n-6) Produced	20:3 (n-6) Produced	% Conversion	18:3 (n-3) Incorporated	18:4 (n-3) Produced	20:4 (n-3) Produced	% Conversion
Control	pYX242 + pYES2	6.46	0	0	0	13.26	0	0	0
pRSP5	pRSP1 (Δ6) + pRAE73-A3 (human elongase)	4.62	1.95	0.8	37.3	7.00	2.47	1.20	34.39
pRSP8	pRSP1 (Δ6) + pRPB2 (M. alpina elongase)	4.08	2.31	0.63	41	5.93	2.01	0.85	32.53

50 μM substrate added

Key:

- 18:2 (n-6) = Linoleic acid
- 18:3 (n-3) = α-Linolenic acid
- 18:3 (n-6) = γ-Linolenic acid
- 18:4 (n-3) = Stearidonic acid
- 20:3 (n-6) = Dihomo-γ-linolenic acid
- 20:4 (n-3) = Eicosatetraenoic acid

% Conversion = 
$$\frac{[\% \text{ Product 1} + \% \text{ Product 2}]}{[\% \text{ substrate} + \% \text{ Product 1} + \% \text{ Product 2}]}$$

Table 4  
Fatty Acid as a Percentage of Total Lipid Extracted from Yeast

Clone	Plasmid in yeast (enzyme)	18:3 (n-6) Incorporated	20:3 (n-6) Produced	20:4 (n-6) Produced	% Conversion	18:4 (n-3) Incorporated	20:4 (n-3) Produced	20:5 (n-3) Produced	% Conversion
Control	pYX242 + pYES2	8.17	0	0	0	5.61	0	0	0
pRSP7	pRSP3 (Δ5) + pRAE73-A3 (human elongase)	6.25	2.30	1.63	38.6	4.12	1.98	1.56	46.2
pRSP10	pRSP3 (Δ5) + pRPB2 (M. alpina elongase)	7.00	2.07	1.35	32.82	4.66	1.50	1.61	40.02

50 μM substrate added

Key:

- 18:3 (n-6) = γ-Linolenic acid
- 18:4 (n-3) = Stearidonic Acid
- 20:3 (n-6) = Dihomo-γ-linolenic acid
- 20:4 (n-6) = Arachidonic Acid
- 20:4 (n-3) = Eicosatetraenoic acid
- 20:5 (n-3) = Eicosapentanoic Acid

% Conversion = 
$$\frac{[\% \text{ Product 1} + \% \text{ Product 2}]}{[\% \text{ substrate} + \% \text{ Product 1} + \% \text{ Product 2}]}$$

Table 5

*Thraustochytrium aureum* (ATCC 34304) Desaturase Expression in Baker's Yeast

CLONE	TYPE OF ENZYME ACTIVITY	% CONVERSION OF SUBSTRATE	
PRTA4	$\Delta 9$	0	(18:0 to 18:1n-9)*
<i>(T. aureum</i> $\Delta 5$ desaturase)	$\Delta 12$	0	(18:1 to 18:2n-6)
	$\Delta 15$	0	(18:2n-6 to 18:3n-3)
	$\Delta 6$	0	(18:2n-6 to 18:3n-6)
	$\Delta 6$	0	(18:3n-3 to 18:4n-3)
	$\Delta 5$	23.7	(20:3n-6 to 20:4n-6)
	$\Delta 17$	0	(20:4n-6 to 20:5n-3)
	$\Delta 19$	0	(22:4n-6 to 22:5n-3)
	$\Delta 4$	0	(22:4n-6 to 22:5n-6)
	$\Delta 4$	0	(22:5n-3 to 22:6n-3)

\*above endogenous  $\Delta 9$  activity

Table 6  
Fatty Acid as a Percentage of Total Lipid Extracted from Yeast

Clone	18:1* Present	18:2 Produced	18:2(n-6) Incorporated	18:3(n-6) Produced	18:3(n-6) Produced	18:3(n-3) Incorporated	18:4(n-3) Produced	20:3(n-6) Incorporated	20:4(n-6) Produced
PYX242 (control)	32.13	0	8.68	0	0	54.42	0	4.3	0
PRTA4 (Δ5)	29.67	0	11.18	0	0	9.93	0	21.94	6.84

50 μM substrate added  
\*18:1 is an endogenous fatty acid in yeast

- Key:
- |           |                          |
|-----------|--------------------------|
| 18:1      | =Oleic acid              |
| 18:2(n-6) | =Linoleic acid           |
| 18:3(n-3) | =α-Linolenic acid        |
| 18:3(n-6) | =γ-Linolenic acid        |
| 18:4(n-3) | =Stearidonic acid        |
| 20:3(n-6) | =Dihomo-γ-linolenic acid |
| 20:4(n-6) | =Arachidonic acid        |

Table 7  
Fatty Acid as a Percentage of Total Lipid Extracted from Yeast

Clone	20:3 Incorporated	20:4 Produced	22:4 Produced	Conversion to products
PYX242/ PYES2 (control)	41.98	0	0	0
PRTA4(Δ5)/ PRAE73-A3 (human elongase)	15.59	4.2	6.28	16.7

100 μM substrate added  
\*18:1 is an endogenous fatty acid in yeast

- Key:
- γ-18:3 =γ-Linolenic acid
  - 20:3 =Dihomo-γ-linolenic acid
  - 20:4 =Arachidonic acid
  - 22:4 =Adrenic acid







Table 10  
Fatty Acid as a Percentage of Total Lipid Extracted from Yeast

Clone	18:3n-3	18:4n-3 Produced	20:3n-6	20:4n-6 Produced	percent conversion	20:4n-3	20:5n-3 Produced	percent conversion
PYX242 (control)	2.65	0	7.2	0	0	11.36	0.38	3.2
PRIG1 (Δ5)	2.28	0	12.17	10.12	45.4	8.43	12.59	59.75

50 uM substrate added

Key:

- 18:3n-3
- =αLinolenic acid
- 18:4n-3
- =Stearidonic acid
- 20:3n-6
- =Dihomo-γ-linolenic acid
- 20:4n-6
- =Arachidonic acid
- 20:4n-3
- =n-3 Eicosatetraenoic acid
- 20:5n-3
- =Eicosapentaenoic acid

percent conversion=  $\frac{\text{product}}{\text{substrate} + \text{product}} \times 100$

CLAIMS:

1. An isolated nucleic acid fragment comprising or  
complementary to a nucleotide sequence encoding a  
polypeptide having desaturase activity, wherein the amino  
acid sequence of said polypeptide has at least 50%  
sequence identity to an amino acid sequence selected from  
the group consisting of SEQ ID NO:20, SEQ ID NO:29, SEQ ID  
NO:31, SEQ ID NO:33 and SEQ ID NO:35.

2. An isolated nucleotide sequence comprising  
or complementary to at least about 50% of the nucleotide  
sequence selected from the group consisting of SEQ ID  
NO:13, SEQ ID NO:19, SEQ ID NO:28, SEQ ID NO:30, SEQ ID  
NO:32 and SEQ ID NO:34.

3. The isolated nucleotide sequence of claim 2 wherein  
said sequence is selected from the group consisting of SEQ  
ID NO:13, SEQ ID NO:19, SEQ ID NO:28, SEQ ID NO:30, SEQ ID  
NO:32 and SEQ ID NO:34.

4. The isolated nucleotide sequence of claim 2 or 3  
wherein said sequence encodes a functionally active  
desaturase which utilizes a polyunsaturated fatty acid as  
a substrate.

5. The nucleotide sequence of claim 4 wherein SEQ ID NO:13  
and SEQ ID NO:19 are derived from *Saprolegnia diclina*.

6. The nucleotide sequence of claim 5 wherein  
SEQ ID NO:28, SEQ ID NO:30 and SEQ ID NO:32 are derived  
from *Thraustochytrium aureum*.

7. The nucleotide sequence of claim 6 wherein SEQ ID NO:34 is derived from *Isochrysis galbana*.

8. A purified polypeptide encoded by said nucleotide sequence of claim 1, 2 or 3.

9. A purified polypeptide which desaturates polyunsaturated fatty acids at carbon 5 and has at least about 50% amino acid identity to an amino acid sequence selected from the group consisting of SEQ ID NO:20, SEQ ID NO:29, SEQ ID NO:31 and SEQ ID NO:35.

10. A purified polypeptide which desaturates polyunsaturated fatty acids at carbon 6 and has at least about 50% amino acid identity to an amino acid sequence selected from the group consisting of SEQ ID NO:33 and SEQ ID NO:14.

11. A method of producing a desaturase comprising the steps of:

- a) isolating a nucleotide sequence selected from the group consisting of SEQ ID NO:13, SEQ ID NO:19, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32 and SEQ ID NO:34;
- b) constructing a vector comprising: i) said isolated nucleotide sequence operably linked to ii) a regulatory sequence;
- c) introducing said vector into a host cell for a time and under conditions sufficient for expression of said desaturase.

12. A vector comprising: a) a nucleotide sequence selected from the group consisting of SEQ ID NO:13, SEQ ID NO:19,

SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32 and SEQ ID NO:34, operably linked to b) a regulatory sequence.

13. A host cell comprising said vector of claim 12.

14. A mammalian cell comprising said vector of claim 12, wherein expression of said nucleotide sequence of said vector results in production of altered levels of AA, EPA, GLA or STA, when said cell is grown in a culture media comprising at least one fatty acid selected from the group consisting of LA, ALA, DGLA and EPA.

15. A plant cell, plant or plant tissue comprising said vector of claim 12, wherein expression of said nucleotide sequence of said vector results in production of a polyunsaturated fatty acid by said plant cell, plant or plant tissue.

16. The plant cell, plant or plant tissue of claim 15 wherein said polyunsaturated fatty acid is selected from the group consisting of AA, EPA, GLA and STA.

17. One or more plant oils or acids expressed by said plant cell, plant or plant tissue of claim 15.

18. A transgenic plant comprising said vector of claim 12, wherein expression of said nucleotide sequence of said vector results in production of a polyunsaturated fatty acid in seeds of said transgenic plant.

19. A method for producing a polyunsaturated fatty acid comprising the steps of:



a) isolating a nucleotide sequence selected from the group consisting of SEQ ID NO:19, SEQ ID NO:28, SEQ ID NO:30 and SEQ ID NO:34;

5 b) constructing a vector comprising said isolated nucleotide sequence;

c) introducing said vector into a host cell for a time and under conditions sufficient for expression of  $\Delta 5$ -desaturase enzyme; and

10 d) exposing said expressed  $\Delta 5$ -desaturase enzyme to a substrate polyunsaturated fatty acid in order to convert said substrate to a product polyunsaturated fatty acid.

20. The method according to claim 19, wherein said  
15 substrate polyunsaturated fatty acid is DGLA or 20:4n-3 and said product polyunsaturated fatty acid is AA or EPA, respectively.

21. The method according to claim 19 further comprising  
20 the step of exposing said product polyunsaturated fatty acid to an elongase in order to convert said product polyunsaturated fatty acid to another polyunsaturated fatty acid.

22. The method according to claim 21 wherein said product  
25 polyunsaturated fatty acid is AA or EPA and said another polyunsaturated fatty acid is adrenic acid or (n-3)-docosapentaenoic acid, respectively.

23. The method of claim 21 further comprising the  
30 step of exposing said another polyunsaturated fatty acid to an additional desaturase in order to convert said

another polyunsaturated fatty acid to a final polyunsaturated fatty acid.

24. The method of claim 23 wherein said final  
5 polyunsaturated fatty acid is (n-6)-docosapentaenoic acid or docosahexaenoic (DHA) acid.

25. A method for producing a polyunsaturated fatty acid comprising the steps of:

- 10 a) isolating a nucleotide sequence selected from the group consisting of SEQ ID NO:13 and SEQ ID NO:32;  
b) constructing a vector comprising said isolated nucleotide sequence;  
c) introducing said vector into a host cell for a time and  
15 under conditions sufficient for expression of  $\Delta 6$ -desaturase enzyme; and  
d) exposing said expressed  $\Delta 6$ -desaturase enzyme to a substrate polyunsaturated fatty acid in order to convert said substrate to a product polyunsaturated fatty acid.

20 26. The method according to claim 25, wherein said substrate polyunsaturated fatty acid is LA or ALA and said product polyunsaturated fatty acid is GLA or STA, respectively.

25 27. The method according to claim 25 further comprising the step of exposing said product polyunsaturated fatty acid to an elongase in order to convert said product polyunsaturated fatty acid to another polyunsaturated  
30 fatty acid.

28. The method according to claim 27 wherein said product polyunsaturated fatty acid is GLA or STA and said another polyunsaturated fatty acid is DGLA or ETA, respectively.

5 29. The method of claim 27 further comprising the step of exposing said another polyunsaturated fatty acid to an additional desaturase in order to convert said another polyunsaturated fatty acid to a final polyunsaturated fatty acid.

10 30. The method of claim 29 wherein said final polyunsaturated fatty acid is AA or EPA.

15 31. A composition comprising at least one polyunsaturated fatty acid selected from the group consisting of said product polyunsaturated fatty acid produced according to the method of claim 19 or 25, said another polyunsaturated fatty acid produced according to the method of claim 21 or 27, and said final  
20 polyunsaturated fatty acid produced according to the method of claim 23 or 29.

25 32. The composition of claim 31 wherein said product polyunsaturated fatty acid is at least one polyunsaturated fatty acid selected from the group consisting of AA, EPA, GLA and STA.

30 33. The composition of claim 31 wherein said another polyunsaturated fatty acid is at least one polyunsaturated fatty acid selected from the group consisting of adrenic acid, (n-3)-docosapentaenoic acid, DGLA and EPA.

34. The composition of claim 31 wherein said final  
polyunsaturated fatty acid is at least one polyunsaturated  
fatty acid selected from the group consisting of (n-6)-  
docosapentaenoic acid, docosahexaenoic (DHA) acid, AA and  
EPA.

35. A method of preventing or treating a condition in a  
patient caused by insufficient intake of polyunsaturated  
fatty acids comprising administering  
to said patient said composition of claim 31 in an amount  
sufficient to effect said prevention or treatment.

Figure 1  
Fatty Acid Biosynthetic Pathway

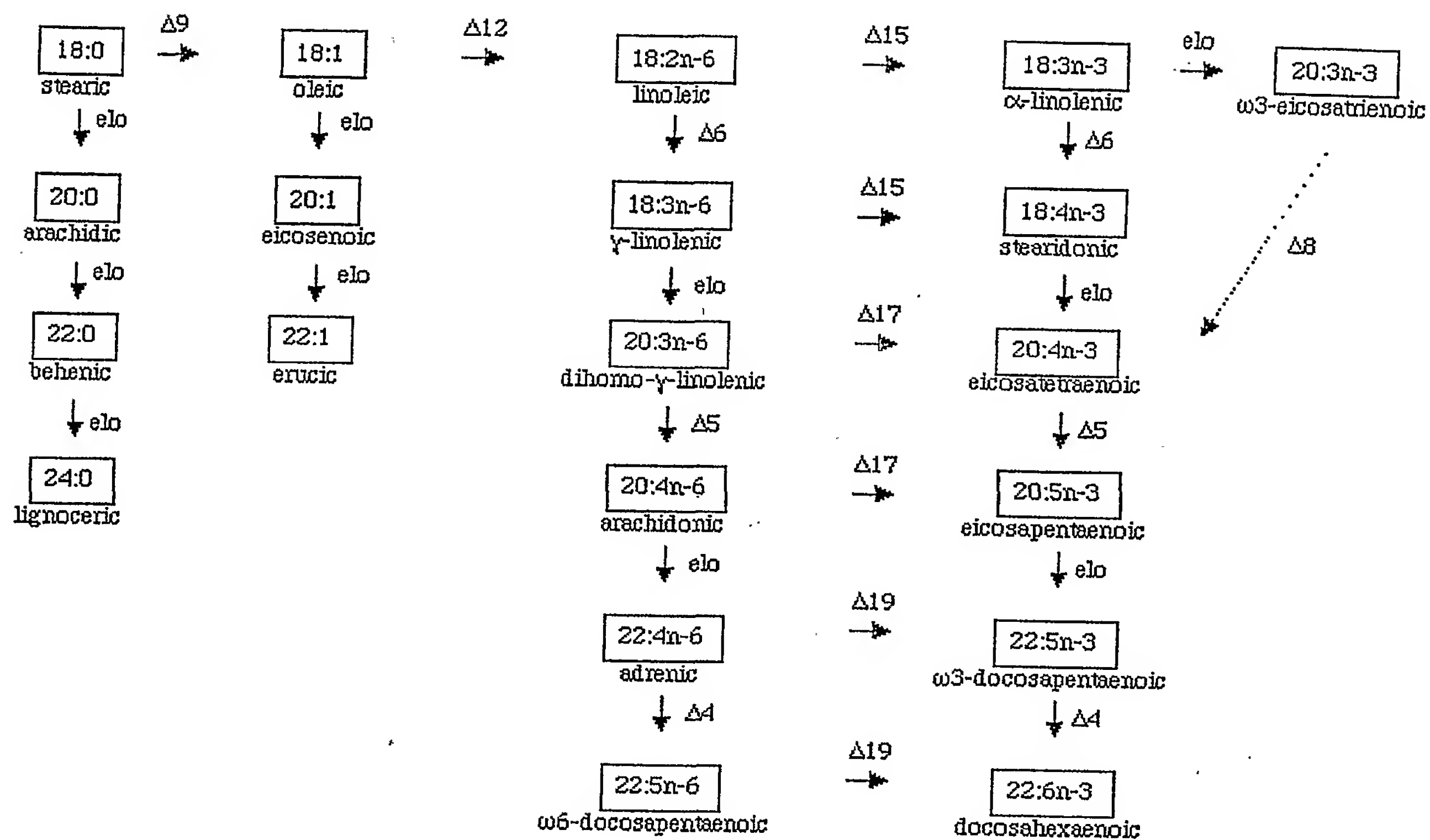


Figure 2

Gene Sequence of Delta 6-Desaturase from *Nyctatherus arizonae* (ATCC 56851)

```

1   ATGGTCCAGG GGC AAAAGGC CGAGAAGATC TCGTGGGCGA CCATCCGTGA
51  GCACAACCGC CAGACAACG CGTGGATCGT GATCCACCAC AAGGTGTACG
101 ACATCTCGGC CTTTGAGGAC CAOCGGGGCG GCGTCGTCTT GTTCACSCAG
151 GCCGGCGAAG ACGCGACCGA TGGTTTCGCT GTCTTCCAOC CGAGCTCGGC
201 GCTCAAGCTC CTCGAGCAGT ACTACGTCGG CGACGTCGAC CAGTCGACGG
251 CGGCGCTCGA CAGTTCGATC TCGGACGAGG TCAAGAAGAG CCAATCGGAC
301 TTCAATTGCGT CGTACCGCAA GCTGCGCCTT GAAATCAAGC GCCTCGGCTT
351 GTACGACTCG AGCAAGCTCT ACTACCTCTA CAAGTGCAGC TCGACGCTGA
401 GCAATTGCGCT TGTGTCGGCG GCCATTTGCC TCCACTTTGA CTCGACGGCC
451 ATGTACATGG TCGCGGCTGT CATCCTTGGC CTCTTTTACC AGCAGTGCGG
501 CTGGCTCGCC CATGACTTTC TGCACCACCA AGTGTTTGAG AACCACTTGT
551 TTGGCGACCT CGTCGGCGTC ATGGTCGGCA ACCTCTGGCA GGGCTTCTCG
601 GTGCAATGGT GGAGAACAA GCACAACACG CACCATGCGA TCCCCAAGCT
651 CCAACGACAG CCGAGATCG CCTTCCACGG CGACCCGGAC ATTGACACGA
701 TGCCGATTCT CGGTGCTCG CTCAAGATGG CGCAGCACGC GGTGACTCG
751 CCCGTCGGGC TCTTCTTCAT GCGCTACCAA GCGTACCTGT ACTTTCCCAT
801 CTTGCTCTTT GCGGTATCT CGTGGGTGAT CCAATCGGCG ATGTACGCTT
851 TCTACAACGT TGGGCCCGGC GGCACCTTTG ACAAGGTCCA GTACCCGCTG
901 CTCGAGCGCG CCGGCTCCT CCTCTACTAC GGCTGGAAOC TCGGCTTGT
951 GTACGACGCC AACATGTCGC TGCTCCAAGC GGCTGCGTTC CTCTTTGTGA
1001 GCCAGGCGTC GTGCGGCTTC TTCTCGCGA TGGTCTTTAG CGTCGGCCAC
1051 AACGGCATGG AGGTCTTTGA CAAGGACAGC AAGCCGATT TTTGGAAGCT
1101 GCAGTGCTC TCGACGCGCA ACCTGACGTC GTGCTCTGCG ATCGACTGGT
1151 TCATGGGCGG CCTCAACTAC CAGATCGACC ACCACTTGTT CCCGATGGTG
1201 CCCCGGCACA ACCTCCCGGC GCTCAACGTC CTCGTCAAGT CGCTCTGCAA
1251 GCAGTACGAC ATCCCATACC ACGAGACGGG CTTCAATCGG GGCATGGGCG
1301 AAGTCTGCTG GCACCTCGAG CGCATCTCGA TCGAGTTCTT CAAGGAGTTT
1351 CCCGCCATGT AA

```



**Figure 3**Amino Acid Sequence of Delta 6- Desaturase from *Syngnathus ferox* (ATCC 56851)

```
1  MYQSQKAEKI SWATIREHNR QDNAMIVIH KVIDISAFED HBGGVMEFQ
51  AGEDATDAFA VEHPSALKL LEQYVVDVD QSTAAVDTSI SDEVKKSQSD
101 FIASYRKLRL EVKRIGLYDS SKLYYLYKCA STLSIALYSA AICLHEDSTA
151 MYMVAAYILG LFYQCCGNLA HDELMHQVEE NHLEGDIVGV MVENLMOGES
201 YQWMIKKNHT HHAIPNLHAT PEIAFHGDDP IDTMBILAMS LKMAQHAVDS
251 PVGLEEFMRVQ AYLYEPILE ARISHVIQSA MYAFYNVEPG GTEDKVQYPL
301 IERAGLLLYY GWNIGLYYAA NMSLLQAAAE LFVSQLSCGL FLAMVESVGH
351 NGMEVEIKDS KEDFNKIQYL STRNVTSGLH IDWFMSSGLY QIDHHLEPMV
401 ERHNLPALNY EVKSLCKQYD IPYHETGETA GMAEVVWHE RISIEEFKEF
451 PAM*
```

**Figure 4**Gene Sequence of Delta 5- Desaturase from *Styromonas danicus* (ATCC 56851)

```

1  ATGGCCCCGC AGACGGAGCT CCGCCAGCGC CACGCCGCCG TCGCCGAGAC
51  GCCGGTGGCC GGCAAGAAGG CCTTTACATG GCAGGAGGTC GCGCAGCACA
101 ACACGGCGGC CTCGGCCTGG ATCATTATCC GCGGCAAGGT CTACGACGTG
151 ACCGAGTGGG CCAACAAGCA CCGCGCGGGC GCGAGATGG TGGTGTGCA
201 CGCCGATCGC GAGGCCACCG ACACGTTTCA CTCGTACCAC CCGTTCAGCG
251 ACAAGGCCGA GTCGATCTTG AACAAATATG AGATTGGCAC GTTCACGGGC
301 CCGTCCGAGT TTCCGACCTT CAAGCCGGAC ACGGGCTTCT ACAAGGAGTG
351 CCGCAAGCGC GTTGGCGAGT ACTCAAGAA GAACAACCTC CATCCGAGG
401 ACGGCTTCCC GGGCCTCTGG CGCATGATGG TCGTGTTTGC GGTCCCGGGC
451 CTCGCCTTGT ACGGCATGCA CTTTTCGACT ATCTTTGCGC TGCAGCTCGC
501 GGCCGCGGGC CTCTTTGGCG TCTGCCAGGC GCTGCCGCTG CTCCACGTCA
551 TGCACGACTC GTCGACGCG TCGTACACCA ACATGCCGTT CTTCATTAC
601 GTCGTGCGCC GCTTTGCCAT GGAATGGTTT GCCGGCGGCT CGATGGTGTG
651 ATGGCTCAAC CAGCACGTCT TGGGCCACCA CATCTACAG AACGTCCGGG
701 GCTCGGACCC GGATCTTCCG GTCAACATGG ACGGCGACAT CCGCCGATC
751 GTGAACCGCC AGGTGTTCCA GCCCATGTAC GCATTCCAGC ACATCTACCT
801 TCCGCCGCTC TATGGCGTGC TTGGCCTCAA GTTCCGCATC CAGGACTTCA
851 CCGACACGTT CGGCTCGCAC ACGAACGGCC CGATCCGCGT CAACCCGCAC
901 GCGCTCTCGA CGTGGATGGC CATGATCAGC TCCAAGTCGT TCTGGGCCTT
951 CTACCGCGTG TACCTTCCGC TTGCCGTGCT CCAGATGCCC ATCAAGACGT
1001 ACCTTGCGAT CTTCTTCTC GCGGAGTTTG TCACGGGCTG GTACCTCGCG
1051 TTCAACTTCC AAGTAAGCCA TGCTTCGACC GAGTGGGCT ACCCATGCGG
1101 CGACGAGGCC AAGATGGCGC TCCAGGACGA GTGGGCGATC TCGCAGGTCA
1151 AGACGTGCGT CGACTACGCC CATGGCTCGT GGATGACGAC GTTCCTTGCC
1201 GCGCGGCTCA ACTACAGGT CGTGCACCAC TTGTTCCCA GCGTGTGCA
1251 GTACCACTAC CCGGCGATCG CCCCCATCAT GGTGACGTC TGCAAGGAGT
1301 ACAACATCAA GTACGCCATC TTGCCGACT TTACGGCGGC GTTCGTTGCC
1351 CACTTGAGGC ACCTCCGCAA CATGGGCCAG CAGGGCATCG CCGCCACGAT
1401 CCACATGGGC TAA

```

**Figure 5**

Amino Acid Sequence of Delta 5- Desaturase from *Staphylococcus aureus* (ATCC 56851)

```
1   MAPQTE LRQR HAAYAETPYA GKKE THQEV AQHNTAASAW IIRGKY YDV
51  TEWANKHPGG REMYLHAGR EATDTEDSYH EF SDKAESIL NKYEIGTF TG
101 PSEF PTEKED TGE YKECRKR YGE YEKCNIL HPQDGEFGLW RMMVYFAYAG
151 LALYGMHE ST IFATQLAAAA LFGVCQALPL LHYMHDSHA SYTNMEEFHY
201 VVGREAMDHE AGG SMVSMIN QHVVGHILYT NVAGSDRDLR VNMDGDIRRI
251 VNRQVE QPMY AFQHIYLPPL YGVIG IKERI QDE TDTEGSH TNG PIRYNBH
301 ALSTMMAMIS SKSEMAFYRY YLPPLAVLQMP IKT YLAIEF L AEFY TGV YLA
351 ENFQYSHVST ECG YPCGDEA KMAIQDE MAY SQVKTSVDYA HG SHMTTELA
401 GADNYQVYHH LEPSVSDYHY PAIAPITVDV CKE YNCKYAI LPDE TAAFYA
451 HLKHLRNMSQ QGLAATHMG *
```

## Figure 6

Gene Sequence of Delta 5- Desaturase from *Thermotoga sibirica* (ATCC 34304)

```

1   ATGGGACGCG GCGGCGAAGG TCAGGTGAAC AGCGCGCAGG TGCCACAAGG
51  CCGTGCGGGA ACGCGAAAGA CGATCCTGAT CGAGGGCGAG GTCTACGATG
101 TCACCAACTT TAGGCACCCC GGCGGGTCGA TCATCAAGTT TCTCAGGACC
151 GACGGCACCG AGGCTGTGGA CGCGACGAAC GCGTTTCGCG AGTTTCACTG
201 CCGGTGCGGC AAGGCGGAAA AGTACCTCAA GAGCCTGCCC AAGCTCGGCG
251 CGCGAGCAA GATGAAGTTT GACGCCAAGG AGCAGGCCCG GCGCGACGCG
301 ATCAGCGGAG ACTACGTCAA GCTGCGCGAG GAGATGGTGG CCGAGGGCCT
351 CTTCAAGCCC GCGCCCCCTC ACATTGTCTA CAGGTTTGCG GAGATCGCAG
401 CCCTGTTCGC GGCTCGTTC TACCTGTTTT CGATGCGCGG AAACGTGTTC
451 GCCACGCTCG CGCCATCGC AGTCGGGGGC ATCGCGCAGG GCGCTGCGG
501 CTGGCTCATG CACGAGTGCG GACACTTCTC GATGACCGGG TACATCCGCG
551 TTGACGTGCG CCTGCAGGAG CTGGTGACG GCGTGGGGTG CTGATGTGCG
601 GCGAGCTGGT GGCGCGTTCA GCACAACAAG CACCACGCGA CCGCGAGAA
651 ACTCAAGCAC GACGTGACG TCGACACCCG GCGCTCGTT GCGTTCAACG
701 AGAAGATCGC CGCCAAGGTG CGCCCCGGCT CGTCCAGGC CAAGTGGCTC
751 TCGGCGCAGG CGTACATTTT TGGCCGGTG TCCTGCTTCC TGTTGGTCT
801 CTTCTGGACC CTGTTTCTGC ACCGCGCGCA CATGCCGCGC ACGAGCCACT
851 TTGCTGAGAT GGCGCCGTC GCGGTGCGCG TCGTGGGCTG GGCGGCGCTC
901 ATGCACTCGT TCGGTTACAG CGGAGCGAC TCGTTCGGTC TCTACATGGC
951 CACCTTTGGC TTTGGCTGCA CCTACATCTT CACCAACTTT GCGGTGAGCC
1001 ACACGCACCT CGACGTCAAC GAGCCGACG AGTTCCTGCA CTGGGTGAG
1051 TACGCCGCGC TGCACACGAC CAACGTGTCC AACGACTCGT GGTTCATCAC
1101 CTGGTGGATG TCGTACCTCA ACTTTCAGAT CGAGCACCAC CTCTTTCGT
1151 CGCTGCCCCA GCTCAACGCC CGCGCGTTCG CCGCGCGCT CCGCGCCCTC
1201 TTCGAGAGC ACGGCATGGC TTACGACGAG CGCCCGTACC TTACGCGCT
1251 TGGGACACG TTTGCCAACC TGCACGCCGT GGGCCAAAAC GCGGCGCAGG
1301 CGGCGCCAA GGCGCTTAG

```

## Figure 7

Amino Acid Sequence of Delta 5- Desaturase from *Thiomargarita* (ATCC 34304)

```

1  MERGGEGQVN SAQVAQGGAE TRKTILIEGE YDYTNERHP GGSIIKE LTT
51  DGTEAYDATN AEREENCRSG KAELYKSLP KLGAPSQMCF DAKEQARRDA
101 ITRDYKLERE EMVAEGLEKP ADLHIVYREA ELAALFAASE YLESMRGNYE
151 ATLALAYGG IAGRCGNLM HECGHE SMTG YIPLDYRLQE LYYGYGC SMS
201 ASHWRVQHNK HHATPQKLKH DYDEDTLP LY AENEKIARKV RPSSEQAKHL
251 SAQAYIEAPV SCFLVGLEHT LFIHPRHMER TSHFAEMABV AVRYYGMARL
301 MHSEGYSQSD SEGLYMATEG EGCTYIETNE AVSHTHEDYT E PDEE LHWVE
351 YALHTTNY S NDSMEITHM SYLNEQIEHH LFPSELPQLNA PRYAPRYRAL
401 FEKHGMAYDE RPYLTALGDT FANLHAYGQN AGQAANKAA

```

Figure 8

1 GAATTCACCA TGGGTCGCGG AGCACAGGGA GAGCCAAGGC AGGCCACAGA  
51 GCTGAAGAGC AGCCCAAGTG AGCAGCGTAA GGTGTTGCTC ATTGACGGGC  
101 AGCTGTACGA TGCAACCAAC TTCAGGCATC CTGGTGGCTC CATCATCAAA  
151 TATTTGTGCA CCGATGGCAA GGAGGTAGTT GATGCAACCG AAGCGTACAA  
201 GGAGTTCCAC TGCAGATCCT CGAAGGCGGT CAAGTACCTC AACTCCCTGC  
251 CAAAGATCGA CGGCCCAATC AAGTACAAAT ACGACGCAA GGAGCAGGCT  
301 CGCCATGACA AACTCAGAG GGAGTATGTA GCTCTCCGG AACAGCTCGT  
351 CAAAGGAGGA TACTTTGACC CCAGCCCGCT CCACATTATC TACAGATGCG  
401 CCGAGTTGGC AGCCATGTTT GCTCTCTCGT TCTACCTTTT CTCCCTTCAAG  
451 GGTAACGTCA TGGCCACTAT TGCTGCCATC GTGATTGGGG GGTGCGTGCA  
501 GGGTCGTTGT GGGTGGCTCA TGCATGAAGC TGGCCACTAC AGCATGACCG  
551 GAAACATCCC TGTTGACTTG CGCCTTCAAG AGTTTITGTA CGGAATTGGG  
601 TGTGGCATGA GCGGGGCTTG GTGGAGAAGC CAGCACAAACA AGCACACGC  
651 CACCCCCCAA AAGCTCAAGC ATGACGTTGA TTTGGACACT CTTCCTCTTG  
701 TCGCCTGGAA CGAGAAATTT GCCCGTCGG TCAAGCCAGG TAGCTTCCAG  
751 GCAAAGTGGC TTCACTCTCA GGGATACATC TTTGCCCCAG TCTCCTGCCT  
801 TCTCGTTGGT CTCTTCTGGA CTTTGTACTT GCATCCTCGC CACATGATCC  
851 GCACCAAGCG CAACTTCGAG ATATTTTCTG TCGCTCTGCG CTACGTATGC  
901 TGGTTCTCGC TTTCTTTTGG CATGGGCTAC ACTGTCGGAG AGTCTCTGGG  
951 TCTCTATGTG CTTACTTTTG GACTTGGCTG TACCTACATC TTTACGCATT  
1001 TTGCTGTAAAG CCACACCCAC TTGCCAGTGT CCGAGGAGGA CGAGTACCTG  
1051 CACTGGGTGG AGTACGCTGC GCTGCACACC ACGAACGTTG CCATCGACTC  
1101 GTACGTTGTC ACCTGGCTGA TGAGCTACCT CAACTTTCAG ATCGAGCACC  
1151 ACTTGTTCCT TTTGCTGCCC CAGTTCCGCC ACCCTGCAAT CTCCTCTCGC  
1201 GTCAAGAAAC TTTTCGAGGA CAATGGTCTG GTATACGACG CCCGCTCATA  
1251 CGTCCAGGCG CTCAAGGATA CCTTCGGCAA CCTACACGAA GTGGGCGTCA  
1301 ACGCTGGCCA AGCTGCCAAG AGCGAGTAAG ATCTCGAG

Start/stop underlined



Figure 9

1 MGRGAQGEPR QATELKSSPS EQRKVLLIDG QLYDATNERH PGGSIKYL  
51 TDGKEVVDAT EAYKEFHCRS SKAVKYNSL PKIDGPIKYK YDAKEQARHD  
101 KITREYVALR EQIVKEGYFD PSLHIIYRC AELAAMEFALS FYLFSEKGNV  
151 MATIAAIVIG GCVQGRCGWL MHEAGHYSMT GNIPVDLRHQ EFLYGIGCGM  
201 SGAWWRSQHN KKHATPQKLK HDVDLDTLPL VAWNEKIARR VKPGSEQAKW  
251 LHLQGYIFAP VSCLLVGLEW TLYLHPRHMI RTKRNFEEIFS VALRYVCWFS  
301 LLLSMGYTVG ESLGLYVLTF GLGCTYIFTH FAVSHTHLPV SEEDEYLHWV  
351 EYAAALHTTNV AIDSYYVTWL MSYLNFEQIEH HLEPCCPQFR HPAISSRVKK  
401 LFEDNGLVYD ARSYVQALKD TFGNLHEVGV NAGQAACKSE

Figure 10

1 CCATGGGCGG CGGCGGCGAG AAAAGCGAGG TGGACCAGGT GCAGCCACAA  
51 AAGACCGAGC AGCTCCAGAA GGCCAAGTGG GAGGATGTTG TTCGCATCAA  
101 TGGAGTCGAA TACGACGTCA CGGACTATCT CAGAAAACAC CCTGGTGGCA  
151 GCGTGATCAA GTACGGGCTT GCCAACACCG GCGCTGATGC CACGTCCCTC  
201 TTTGAAGCGT TCCACATGCG CTCAAAGAAG GCTCAGATGG TGCTCAAGTC  
251 TCTCCCAAAG CGTGCTCCGG TCCTCGAGAT CCAGCCAAAC CAGCTTCCAG  
301 AGGAGCAGAC CAAGGAGGCG GAGATGCTGC GTGATTTTAA AAAATTGAG  
351 GATGAGATTG GCCGGGATGG ATTGATGGAA CCTTCCTTCT GGCATCGCGC  
401 TTACAGATTG TCAGAGCTTG TAGGTATGTT CACGCTCGGC CTCTACCTCT  
451 TCTCGTTAAA CACTCCTCTG TCTATTGCTG CTGGTGTCTT CGTCCACGGT  
501 CTCCTTGGTG CATTCTGTGG ATGGTGCCAG CATGAGGCAG GCCACGGCTC  
551 CTTTTTTTAC AGCCTTTGGT GGGCAAGCG GTACAGGCC ATGTTGATCG  
601 GGTTTGGTCT AGGAACATCC GCGACATGT GGAACATGAT GCACAACAAG  
651 CATCATGCTG CCACCCAAA GGTTCATCAC GACCTTGACA TTGACACAAC  
701 TCCTTTTGTA GCTTTCTTCA AACTGTCATT TGAGAAAAAC AGATGGAAGG  
751 GCTTTTCCAA GGCTTGGGTC CGCTTTCAGG CTTTCACGTT CATTCCTGTC  
801 ACCAGCGGCA TGATCGTCAAT GCTGTTCTGG CTGTTTTTTC TCCACCCCTCG  
851 CCGCGTCGTT CAAAAGAAGA ACTTTGAGGA GGTTTTTTGG ATGCTGTCTGA  
901 GCCACATTGT GCGCACCTAT CTCTTCCACC TTGTGACCCG CTGGGAGAGC  
951 CTCGCTGCAT GCTACCTTGT TGGGTATTGG GCGTGCAATG GGTGTCCGG  
1001 TATGTATTG TTTGGCCACT TTTGCTCTC CCACACTCAT ATGGACATTG  
1051 TGGAGGCGGA CGTGCAATAAG AACTGGGTCA GGTACGCTGT TGACCACT  
1101 GTTGACATCA GCCCATCCAA CCGCTCGTG TGCTGGGTCA TGGTTACCT  
1151 CAACATGCAG ACCATCCACC ACTTGTGGCC TGCCATGCCC CAGTACCACC  
1201 AGGTCGAGGT CTCACGCCGC TTTGCCATCT TCGCCAAAA ACACGGCCTC  
1251 AACTACCGCG TCGTCTCTTA CTTTGAGGCT TGGCGCTGA TGCTCCAAA  
1301 TCTTGCTGAC GTCGGTTCCC ACTACCATGA GAACGGTGTG AAGCGGCCC  
1351 CAAAGAAAGC CAAGGCGCAG TAGAAAGCTA T

Start/stop underlined

Figure 11

1 MGRGGEKSEV DQVQPQKTEQ LQAKWEDVV RINGVEYDVT DYLRKHPGGS  
51 VIKYGLANTG ADATSLFEAF HMRSKKAQMV LKSLPKRAPV LEIQPNQLPE  
101 EQTKEAEMLR DEKKFEDEIR RDGLMEPSEW HRAYRLSELV GMFTLGLYLE  
151 SLNTPLSIAA GVLVHGLEGA FCGWCQHEAG HGSEFFYSLWW GKRVDQAMLIG  
201 FGLGTSGDMW NMMHNKHHAA TQKVHHDLDI DTTPFVAFEN TAFEKNRWKG  
251 FSKAWVRFOA FTFIPVTSGM IVMLFWLFEFL HPRRVVQKKN FEEGFWMMLSS  
301 HIVRTYLFHL VTGWESLAAC YLVGYWACMW VSGMYLFGHF SLSTHMDIV  
351 EADVHKNNVR YAVDHTVDIS PSNPLVCWVM GYLNMQTIHH LWPAMPQYHQ  
401 VEVSRREAIF AKKHGLNYRV VSYFEAWRLM LQNLADVGSY YHENGVKRAP  
451 KKAKAQ

Figure 12

pRAT-2a	1	MGRGAQGEPRQATELKSSPSEQRKVL	LDGQLYDA	TNFRHPGGS	I	45
pRAT-2c	1	MGRGAQGEPRQATELKSSPSEQRKVL	LDGQLYDA	TNFRHPGGS	I	45
pRAT-2a	46	IKYLC TDGKEVVDAT	EAYKEFHCRSSKADKY	LN	SLPK	IDGPIKYK
pRAT-2c	46	IKYLC TDGKEVVDAT	EAYKEFHCRSSKADKY	LN	SLPK	IDGPIKYK
pRAT-2a	91	YDAKEQARHDKL	TREYVALREQLVKEGY	FDPSPL	HI	IYRCAE
pRAT-2c	91	YDAKEQARHDKL	TREYVALREQLVKEGY	FDPSPL	HI	IYRCAE
pRAT-2a	136	MFALS FYLFSFKGN	VVA	TI	AA	IVIGGC
pRAT-2c	136	MFALS FYLFSFKGN	VVA	TI	AA	IVIGGC
pRAT-2a	181	GNIPVDLRLQEE	FLYG	IGCGMSG	AWRR	RQH
pRAT-2c	181	GNIPVDLRLQEE	FLYG	IGCGMSG	AWRR	RQH
pRAT-2a	226	DTLPLVAWNEK	IARRVKPGS	FFQAKW	PHL	QGYIFAPV
pRAT-2c	226	DTLPLVAWNEK	IARRVKPGS	FFQAKW	PHL	QGYIFAPV
pRAT-2a	271	TLYLHPRRHM	IRTKRN	FE	IFSV	ALRYVCW
pRAT-2c	271	TLYLHPRRHM	IRTKRN	FE	IFSV	ALRYVCW
pRAT-2a	316	YVLTFFGLGCT	YIFTHFAV	SH	THLPV	SEED
pRAT-2c	316	YVLTFFGLGCT	YIFTHFAV	SH	THLPV	SEED
pRAT-2a	361	AIDSYVVTWLM	SYLNFQ	IEHH	LF	PCCPQFRHP
pRAT-2c	361	AIDSYVVTWLM	SYLNFQ	IEHH	LF	PCCPQFRHP
pRAT-2a	406	GLVYDARSYVQ	AL	KD	TFGN	LHEVGVNAGQ
pRAT-2c	406	GLVYDARSYVQ	AL	KD	TFGN	LHEVGVNAGQ

Figure 13

pRAT-1a	1	MGRGGEKSEVDQVQPQKTEQLQKAKWEDVVRINGVEYDVTDYLR	44
pRAT-1b	1	MGRGGEKSEVDQVQPQKTEQLQKAKWEDVVRINGVEYDVTDYLR	44
pRAT-1a	45	KHPGGSVVKYGLANTGADATSLFEAFHMRSKKAQMVLKSLPKRA	88
pRAT-1b	45	KHPGGSVVKYGLANTGADATSLFEAFHMRSKKAQMVLKSLPKRA	88
pRAT-1a	89	PVLEIQPNQLPEEQTKEAEMLRDFKKFEDEIRRDGLMEPSFWHR	132
pRAT-1b	89	PVLEIQPNQLPEEQTKEAEMLRDFKKFEDEIRRDGLMEPSFWHR	132
pRAT-1a	133	AYRLSELVGMFTLGLYLFSLNTPLSIAAGVLVHGLFGAFCGWCQ	176
pRAT-1b	133	AYRLSELVGMFTLGLYLFSLNTPLSIAAGVLVHGLFGAFCGWCQ	176
pRAT-1a	177	HEAGHGSFFYSLWWGKRVQAMLIGFGLGTSGDMWNMMHNKHHAA	220
pRAT-1b	177	HEAGHGSFFYSLWWGKRVQAMLIGFGLGTSGDMWNMMHNKHHAA	220
pRAT-1a	221	TQKVHHDDLIDTTPFVAFFNATAFEKNRWKGFSAWVRFQAFFTI	264
pRAT-1b	221	TQKVHHDDLIDTTPFVAFFNATAFEKNRWKGFSAWVRFQAFFTI	264
pRAT-1a	265	PVTSGMIVMLFWLFFLHPRRVVQKKNFEEGFWMLSHIVRTYLF	308
pRAT-1b	265	PVTSGMIVMLFWLFFLHPRRVVQKKNFEEGFWMLSHIVRTYLF	308
pRAT-1a	309	HLVTGWESLAACYLVGYWACMWVSGMYLFGHFSLSHTHMDIVEA	352
pRAT-1b	309	HLVTGWESLAACYLVGYWACMWVSGMYLFGHFSLSHTHMDIVEA	352
pRAT-1a	353	DVHKNWVRYAVDHTVDISPSNPLVCWVMGYLNMQTIHHLWPAMP	396
pRAT-1b	353	DVHKNWVRYAVDHTVDISPSNPLVCWVMGYLNMQTIHHLWPAMP	396
pRAT-1a	397	QYHQVEVSRRFAIFAKKHGGLNYRVVSYFEAWRLMLQNLAADVGS	440
pRAT-1b	397	QYHQVEVSRRFAIFAKKHGGLNYRVVSYFEAWRLMLQNLAADVGS	440
pRAT-1a	441	YHENGVKRAPKKAKAQ	456
pRAT-1b	441	YHENGVKRAPKKAKAQ	456

**Figure 14**

```
1  ATGGTGGCAG  GCAAATCAGG  CGCTGCGGCG  CACGTGACTC  ACAGCTCGAC
51  ATTGCCCCGT  GAGTACCATG  GCGCGACCAA  CGACTCGCGC  TCTGAGGCGG
101  CCGACGTCAC  CGTCTCTAGC  ATCGATGCTG  AAAAGGAGAT  GATCATCAAC
151  GGCCGCGTGT  ATGACGTGTC  GTCATTTGTG  AAGCGGCACC  CAGGTGGCTC
201  GGTGATCAAG  TTCCAGCTGG  GCGCCGACGC  GAGCGACGCG  TACAACAAC
251  TTCACGTCCG  CTCCAAGAAG  GCGGACAAGA  TGCTGTATTC  GCTCCCGTCC
301  CGGCCGGCCG  AGGCCGGCTA  CGCCAGGAC  GACATCTCCC  GCGACTTTGA
351  GAAGCTGCGC  CTCGAGCTGA  AGGAGGAGGG  CTACTTCGAG  CCCAACCTGG
401  TGCACGTGAG  CTACAGGTGT  GTGGAGGTTC  TTGCCATGTA  CTGGGCTGGC
451  GTCCAGCTCA  TCTGGTCCGG  GTACTGGTTC  CTCGGCGCGA  TCGTGGCCGG
501  CATTGCGCAG  GGCCGCTGCG  GCTGGCTCCA  GCATGAGGGT  GGGCACTACT
551  CGCTCACCGG  CAACATCAAG  ATCGACCGGC  ATCTGCAGAT  GGCCATCTAT
601  GGGCTTGGCT  GCGGCATGTC  GGGCTGCTAC  TGGCGCAACC  AGCACAACAA
651  GCACCACGCC  ACGCCGCAGA  AGCTCGGGAC  CGACCCCGAC  CTGCAGACGA
701  TGCCGCTGGT  GGCCTTCCAC  AAGATCGTCG  GCGCCAAGGC  GCGAGGCAAG
751  GGCAAGGCGT  GGCTGGCGTG  GCAGGCGCCG  CTCTTCTTTG  GCGGGATCAT
801  CTGCTCGCTC  GTCTCTTTCG  GCTGGCAGTT  CGTGCTCCAC  CCCAACACG
851  CGCTGCGCGT  GCACAATCAC  CTGGAGCTCG  CGTACATGGG  CCTGCGGTAC
901  GTGCTGTGGC  ACCTGGCCTT  TGGCCACCTC  GGGCTGCTGA  GCTCGCTCCG
951  CCTGTACGCC  TTTTACGTGG  CCGTGGGCGG  CACCTACATC  TTCACCAACT
1001  TCGCCGTCTC  GCACACCCAC  AAGGACGTCG  TCCCGCCCAC  CAAGCACATC
1051  TCGTGGGCAC  TCTACTCGGC  CAACCACACG  ACCAACTGCT  CCGACTCGCC
1101  CTTTGTCAAC  TGGTGGATGG  CCTACCTCAA  CTTCCAGATC  GAGCACCACC
1151  TCTTCCCGTC  GATGCCGCAG  TACAACCACC  CCAAGATCGC  CCCGCGGGTG
1201  CGCGCGCTCT  TCGAGAAGCA  CGGGGTCGAG  TATGACGTCC  GGCCATACCT
1251  GGAGTGTTTT  CGGGTCACGT  ACGTCAACCT  GCTCGCCGTA  GGCAACCCGG
1301  AGCACTCCTA  CCACGAGCAC  ACGCACTAG
```



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**Figure 15**

1 MVAGKSGAAA HVTHSSTLPR EYHGATNDSR SEAADVTVSS IDAEKEMIIN  
51 GRVYDVSSFV KRHPGGSVIK FQLGADASDA YNNFHVRSKK ADKMLYSLPS  
101 RPAEAGYAQD DISRDFEKLK LELKEEGYFE PNLVHVSYRC VEVLAMYWAG  
151 VQLIWSGYWF LGAIVAGIAQ GRCGWLQHEG GHYSLTGNIK IDRHLQMAIY  
201 GLGCGMSGCY WRNQHNKHHA TPQKLGTDPD LQTMPLVAFH KIVGAKARGK  
251 GKAWLAWQAP LFFGGIICSL VSFGWQFVLH PNHALRVHNN LELAYMGLRY  
301 VLWHLAFGHL GLLSSLRLYA FYVAVGGTYI FTNFAVSHTH KDVVPPTKHI  
351 SWALYSANHT TNCSDSPFVN WWMAYLNFQI EHHLFPSMPQ YNHPKIAPRV  
401 RALFEKHGVE YDVRPYLECF RVTYVNLLAV GNPEHSYHEH TH

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## SEQUENCE LISTING

<110> Abbott Laboratories  
Mukerji, Pradip  
Huang, Yung-Sheng  
Das, Tapas  
Thurmond, Jennifer M.  
Pereira, Suzette L.

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<223> b = g or c or t/u at position 12

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<223> r = g or a at position 18

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&lt;221&gt; misc\_feature

&lt;222&gt; (30)...(30)

&lt;223&gt; b = g or c or t/u at position 30

&lt;221&gt; misc\_feature

&lt;222&gt; (33)...(33)

&lt;223&gt; y = t/u or c at position 33

&lt;221&gt; misc\_feature

&lt;222&gt; (36)...(36)

&lt;223&gt; y = t/u or c at position 36

&lt;221&gt; misc\_feature

&lt;222&gt; (39)...(39)

&lt;223&gt; h = a or c or t/u at position 39

&lt;221&gt; misc\_feature

&lt;222&gt; (42)...(42)

&lt;223&gt; h = a or c or t/u at position 42

&lt;400&gt; 1

gtbtaygayg tbaccgartg ggtbaagcgy cayccbghg gh

42

&lt;210&gt; 2

&lt;211&gt; 45

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Forward Primer R0835

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&lt;223&gt; y = t/u or c at position 6

&lt;221&gt; misc\_feature

&lt;222&gt; (12)...(12)

&lt;223&gt; y = t/u or c at position 12

&lt;221&gt; misc\_feature

&lt;222&gt; (27)...(27)

&lt;223&gt; y = t/u or c at position 27

&lt;221&gt; misc\_feature

&lt;222&gt; (33)...(33)

&lt;223&gt; y = tu or c at position 33

&lt;221&gt; misc\_feature

&lt;222&gt; (39)...(39)

&lt;223&gt; b = g or c or t/u at position 39

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<223> v = a or g or c at position 7

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<223> r = g or a at position 13

<221> misc\_feature  
<222> (19)...(19)  
<223> r = g or a at position 19

<221> misc\_feature  
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<223> r = g or a at position 34

<221> misc\_feature  
<222> (40)...(40)  
<223> r = g or a at position 40

<221> misc\_feature  
<222> (43)...(43)  
<223> d = a or g or t/u at position 43

<400> 3  
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45

<210> 4  
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<223> y = t/u or c at position 15

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<223> v = a or g or c at position 30

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36

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<223> r = g or a at position 13

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<223> r = g or a at positions 18-19

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<400> 5  
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24

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<223> n = a or g or c or t/u, unknown, or other at  
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<222> (24)...(24)  
<223> y = t/u or c at position 24

<221> misc\_feature  
<222> (27)...(27)  
<223> n = a or g or c or t/u, unknown, or other at  
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30

<210> 7  
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<212> DNA



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&lt;213&gt; Artificial Sequence

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&lt;223&gt; Primer RO923

&lt;400&gt; 7

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29

&lt;210&gt; 8

&lt;211&gt; 30

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer RO899

&lt;400&gt; 8

agcggataac aatttcacac aggaaacagc

30

&lt;210&gt; 9

&lt;211&gt; 30

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer RO939

&lt;400&gt; 9

cgtagtactg ctcgaggagc ttgagcgccg

30

&lt;210&gt; 10

&lt;211&gt; 31

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer RO898

&lt;400&gt; 10

cccagtcacg acgttgtaaa acgacggcca g

31

&lt;210&gt; 11

&lt;211&gt; 45

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer RO951

&lt;400&gt; 11

tcaacagaat tcatgggtcca ggggcaaaag gccgagaaga tctcg

45

&lt;210&gt; 12

&lt;211&gt; 47

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

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&lt;220&gt;

&lt;223&gt; Primer RO960

&lt;400&gt; 12

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47

&lt;210&gt; 13

&lt;211&gt; 1362

&lt;212&gt; DNA

&lt;213&gt; Saprolegnia diclina

&lt;400&gt; 13

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&lt;211&gt; 453

&lt;212&gt; PRT

&lt;213&gt; Saprolegnia diclina

&lt;400&gt; 14

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 1           5           10           15
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          20          25          30
Tyr Asp Ile Ser Ala Phe Glu Asp His Pro Gly Gly Val Val Met Phe
          35          40          45
Thr Gln Ala Gly Glu Asp Ala Thr Asp Ala Phe Ala Val Phe His Pro
          50          55          60
Ser Ser Ala Leu Lys Leu Leu Glu Gln Tyr Tyr Val Gly Asp Val Asp
65          70          75          80
Gln Ser Thr Ala Ala Val Asp Thr Ser Ile Ser Asp Glu Val Lys Lys
          85          90          95
Ser Gln Ser Asp Phe Ile Ala Ser Tyr Arg Lys Leu Arg Leu Glu Val
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Lys Arg Leu Gly Leu Tyr Asp Ser Ser Lys Leu Tyr Tyr Leu Tyr Lys

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145					150					155					160		
Leu	Phe	Tyr	Gln	Gln	Cys	Gly	Trp	Leu	Ala	His	Asp	Phe	Leu	His	His		
				165					170					175			
Gln	Val	Phe	Glu	Asn	His	Leu	Phe	Gly	Asp	Leu	Val	Gly	Val	Met	Val		
			180					185					190				
Gly	Asn	Leu	Trp	Gln	Gly	Phe	Ser	Val	Gln	Trp	Trp	Lys	Asn	Lys	His		
	195						200					205					
Asn	Thr	His	His	Ala	Ile	Pro	Asn	Leu	His	Ala	Thr	Pro	Glu	Ile	Ala		
	210					215					220						
Phe	His	Gly	Asp	Pro	Asp	Ile	Asp	Thr	Met	Pro	Ile	Leu	Ala	Trp	Ser		
225				230						235					240		
Leu	Lys	Met	Ala	Gln	His	Ala	Val	Asp	Ser	Pro	Val	Gly	Leu	Phe	Phe		
			245					250						255			
Met	Arg	Tyr	Gln	Ala	Tyr	Leu	Tyr	Phe	Pro	Ile	Leu	Leu	Phe	Ala	Arg		
			260					265					270				
Ile	Ser	Trp	Val	Ile	Gln	Ser	Ala	Met	Tyr	Ala	Phe	Tyr	Asn	Val	Gly		
	275						280					285					
Pro	Gly	Gly	Thr	Phe	Asp	Lys	Val	Gln	Tyr	Pro	Leu	Leu	Glu	Arg	Ala		
	290					295				300							
Gly	Leu	Leu	Leu	Tyr	Tyr	Gly	Trp	Asn	Leu	Gly	Leu	Val	Tyr	Ala	Ala		
305				310						315					320		
Asn	Met	Ser	Leu	Leu	Gln	Ala	Ala	Ala	Phe	Leu	Phe	Val	Ser	Gln	Ala		
			325						330					335			
Ser	Cys	Gly	Leu	Phe	Leu	Ala	Met	Val	Phe	Ser	Val	Gly	His	Asn	Gly		
			340					345					350				
Met	Glu	Val	Phe	Asp	Lys	Asp	Ser	Lys	Pro	Asp	Phe	Trp	Lys	Leu	Gln		
	355					360						365					
Val	Leu	Ser	Thr	Arg	Asn	Val	Thr	Ser	Ser	Leu	Trp	Ile	Asp	Trp	Phe		
	370					375					380						
Met	Gly	Gly	Leu	Asn	Tyr	Gln	Ile	Asp	His	His	Leu	Phe	Pro	Met	Val		
385				390						395					400		
Pro	Arg	His	Asn	Leu	Pro	Ala	Leu	Asn	Val	Leu	Val	Lys	Ser	Leu	Cys		
			405					410						415			
Lys	Gln	Tyr	Asp	Ile	Pro	Tyr	His	Glu	Thr	Gly	Phe	Ile	Ala	Gly	Met		
			420					425				430					
Ala	Glu	Val	Val	Val	His	Leu	Glu	Arg	Ile	Ser	Ile	Glu	Phe	Phe	Lys		
	435					440						445					
Glu	Phe	Pro	Ala	Met													
	450																

&lt;210&gt; 15

&lt;211&gt; 23

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer R0851

&lt;400&gt; 15

ccatcaagac gtaccttgcg atc

23

&lt;210&gt; 16

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<211> 28  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer R0941

<400> 16  
 gctgaacggg tggtacgagt cgaacgtg 28

<210> 17  
 <211> 42  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer R0953

<400> 17  
 acgagagaat tcatggcccc gcagacggag ctccgccagc gc 42

<210> 18  
 <211> 46  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer R0956

<400> 18  
 aaaagactcg agttagccca tgtggatcgt ggcggcgatg ccctgc 46

<210> 19  
 <211> 1413  
 <212> DNA  
 <213> Saprolegnia diclina

<400> 19  
 atggccccgc agacggagct cgcgcagcgc cagccgcgcg tcgcccagac gccggtggcc 60  
 ggcaagaagg cctttacatg gcaggaggtc gcgcagcaca acacggcggc ctccggcctgg 120  
 atcattatcc gcggaaggt ctacgacgtg accgagtggtg ccaacaagca ccccgggcggc 180  
 cgcgagatgg tgctgctgca cgccggtcgc gaggccaccg acacgttcga ctcgctaccac 240  
 ccgttcagcg acaaggccga gtcgatcttg aacaagtatg agattggcac gttcacgggc 300  
 ccgtccgagt ttccgacctt caagccggac acgggcttct acaaggagtg ccgcaagcgc 360  
 gttggcgagt acttcaagaa gaacaacctc catccgcagg acggcttccc gggcctctgg 420  
 cgcgatgatg tcgtgtttgc ggtcgccggc ctgccttgt acggcatgca cttttcgact 480  
 atctttgcgc tgcagctcgc ggccgcggcg ctctttggcg tctgccaggc gctgccgctg 540  
 ctccacgtca tgcacgactc gtcgcacgcg tcgtacacca acatgccgtt cttccattac 600  
 gtcgtcggcc gctttgccat ggactggttt gccggcggct cgatgggtgc atggctcaac 660  
 cagcacgtcg tgggccacca catctacacg aacgtcgcgg gctcggacc ggatcttccg 720  
 gtcaacatgg acggcgacat ccgcgcgcat gtgaaccgcc aggtgttcca gcccatgtac 780  
 gcattccagc acatctacct tccgccgctc tatggcgtgc ttggcctcaa gttccgcac 840  
 caggacttca ccgacacgtt cggctcgcac acgaacggcc cgatccgcgt caaccgcac 900  
 gcgctctcga cgtggatggc catgatcagc tccaagtcgt tctgggcctt ctaccgcgtg 960  
 taccttccgc ttgccgtgct ccagatgcc atcaagacgt accttgcgat cttcttctc 1020  
 gccgagtttg tcacgggctg gtacctcgcg ttcaacttcc aagtaagcca tgtctcgacc 1080  
 gagtgccggt acccatgcgg cgacgaggcc aagatggcgc tccaggacga gtgggcagtc 1140

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tcgcaggtca agacgtcggc cgactacgcc catggctcgt ggatgacgac gttccttgcc 1200
ggcgcgctca actaccaggt cgtgcaccac ttgttcccca gcgtgtcgca gtaccactac 1260
ccggcgatcg cgcccatcat cgtcgacgtc tgcaaggagt acaacatcaa gtacgccatc 1320
ttgccggact ttacggcggc gttcgttgcc cacttgaagc acctccgcaa catggggccag 1380
cagggcatcg ccgccacgat ccacatgggc taa 1413

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&lt;210&gt; 20

&lt;211&gt; 470

&lt;212&gt; PRT

&lt;213&gt; Saprolegnia diclina

&lt;400&gt; 20

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Thr	Pro	Val	Ala	Gly	Lys	Lys	Ala	Phe	Thr	Trp	Gln	Glu	Val	Ala	Gln	20	25	30	
His	Asn	Thr	Ala	Ala	Ser	Ala	Trp	Ile	Ile	Ile	Arg	Gly	Lys	Val	Tyr	35	40	45	
Asp	Val	Thr	Glu	Trp	Ala	Asn	Lys	His	Pro	Gly	Gly	Arg	Glu	Met	Val	50	55	60	
Leu	Leu	His	Ala	Gly	Arg	Glu	Ala	Thr	Asp	Thr	Phe	Asp	Ser	Tyr	His	65	70	75	80
Pro	Phe	Ser	Asp	Lys	Ala	Glu	Ser	Ile	Leu	Asn	Lys	Tyr	Glu	Ile	Gly	85	90	95	
Thr	Phe	Thr	Gly	Pro	Ser	Glu	Phe	Pro	Thr	Phe	Lys	Pro	Asp	Thr	Gly	100	105	110	
Phe	Tyr	Lys	Glu	Cys	Arg	Lys	Arg	Val	Gly	Glu	Tyr	Phe	Lys	Lys	Asn	115	120	125	
Asn	Leu	His	Pro	Gln	Asp	Gly	Phe	Pro	Gly	Leu	Trp	Arg	Met	Met	Val	130	135	140	
Val	Phe	Ala	Val	Ala	Gly	Leu	Ala	Leu	Tyr	Gly	Met	His	Phe	Ser	Thr	145	150	155	160
Ile	Phe	Ala	Leu	Gln	Leu	Ala	Ala	Ala	Ala	Leu	Phe	Gly	Val	Cys	Gln	165	170	175	
Ala	Leu	Pro	Leu	Leu	His	Val	Met	His	Asp	Ser	Ser	His	Ala	Ser	Tyr	180	185	190	
Thr	Asn	Met	Pro	Phe	Phe	His	Tyr	Val	Val	Gly	Arg	Phe	Ala	Met	Asp	195	200	205	
Trp	Phe	Ala	Gly	Gly	Ser	Met	Val	Ser	Trp	Leu	Asn	Gln	His	Val	Val	210	215	220	
Gly	His	His	Ile	Tyr	Thr	Asn	Val	Ala	Gly	Ser	Asp	Pro	Asp	Leu	Pro	225	230	235	240
Val	Asn	Met	Asp	Gly	Asp	Ile	Arg	Arg	Ile	Val	Asn	Arg	Gln	Val	Phe	245	250	255	
Gln	Pro	Met	Tyr	Ala	Phe	Gln	His	Ile	Tyr	Leu	Pro	Pro	Leu	Tyr	Gly	260	265	270	
Val	Leu	Gly	Leu	Lys	Phe	Arg	Ile	Gln	Asp	Phe	Thr	Asp	Thr	Phe	Gly	275	280	285	
Ser	His	Thr	Asn	Gly	Pro	Ile	Arg	Val	Asn	Pro	His	Ala	Leu	Ser	Thr	290	295	300	
Trp	Met	Ala	Met	Ile	Ser	Ser	Lys	Ser	Phe	Trp	Ala	Phe	Tyr	Arg	Val	305	310	315	320
Tyr	Leu	Pro	Leu	Ala	Val	Leu	Gln	Met	Pro	Ile	Lys	Thr	Tyr	Leu	Ala	325	330	335	
Ile	Phe	Phe	Leu	Ala	Glu	Phe	Val	Thr	Gly	Trp	Tyr	Leu	Ala	Phe	Asn	340	345	350	
Phe	Gln	Val	Ser	His	Val	Ser	Thr	Glu	Cys	Gly	Tyr	Pro	Cys	Gly	Asp				

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	355		360		365										
Glu	Ala	Lys	Met	Ala	Leu	Gln	Asp	Glu	Trp	Ala	Val	Ser	Gln	Val	Lys
	370					375					380				
Thr	Ser	Val	Asp	Tyr	Ala	His	Gly	Ser	Trp	Met	Thr	Thr	Phe	Leu	Ala
385					390					395					400
Gly	Ala	Leu	Asn	Tyr	Gln	Val	Val	His	His	Leu	Phe	Pro	Ser	Val	Ser
			405						410					415	
Gln	Tyr	His	Tyr	Pro	Ala	Ile	Ala	Pro	Ile	Ile	Val	Asp	Val	Cys	Lys
			420					425					430		
Glu	Tyr	Asn	Ile	Lys	Tyr	Ala	Ile	Leu	Pro	Asp	Phe	Thr	Ala	Ala	Phe
		435					440					445			
Val	Ala	His	Leu	Lys	His	Leu	Arg	Asn	Met	Gly	Gln	Gln	Gly	Ile	Ala
	450					455					460				
Ala	Thr	Ile	His	Met	Gly										
465					470										

<210> 21  
 <211> 914  
 <212> DNA  
 <213> Homo sapiens

<400> 21  
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 actagagtaa aaggatgggt tcttctggac aattatatac ccacatttat ctgctctgtc 120  
 atatatattac taattgtatg gctgggacca aaatacatga ggaataaaca gccattctct 180  
 tgccggggga ttttagtggt gtataacctt ggactcacac tgctgtctct gtatatgttc 240  
 tgtgagttag taacaggagt atgggaaggc aaatacaact tcttctgtca gggcacacgc 300  
 accgcaggag aatcagatat gaagattatc cgtgtcctct ggtggtacta cttctccaaa 360  
 ctcatagaat ttatggacac tttcttcttc atcctgcgca agaacaacca ccagatcacg 420  
 gtccctgcacg tctaccacca tgccctcgatg ctgaacatct ggtggtttgt gatgaactgg 480  
 gtccctctgcg gccactctta ttttggtgcc acacttaata gcttcatcca cgtcctcatg 540  
 tactcttact atggtttgtc gtcagtccct tccatgcgtc catacctctg gtggaagaag 600  
 tacatcactc aggggcagct gcttcagttt gtgctgacaa tcatccagac cagctgcggg 660  
 gtcatctggc cgtgcacatt ccctcttggt tgggtgtatt tccagattgg atacattatt 720  
 tccctgattg ctctcttcac aaacttctac attcagacct acaacaagaa aggggcctcc 780  
 cgaaggaaag accacctgaa ggaccaccag aatgggtccg tggctgctgt gaatggacac 840  
 accaacagct tttcacccct ggaaaacaat gtgaagccaa ggaagctgcg gaaggattga 900  
 agtcaaagaa ttga 914

<210> 22  
 <211> 957  
 <212> DNA  
 <213> Mortierella alpina

<400> 22  
 atggagtcga ttgcgccatt cctcccatca aagatgccgc aagatctggt tatggacctt 60  
 gccaccgcta tcggtgtccg ggccgcgccc tatgtcgatc ctctcgaggc cgcgctgggt 120  
 gccaggccg agaagtacat cccacgatt gtccatcaca cgcgtgggtt cctggctcgc 180  
 gtggagtcgc ctttggcccg tgagctgccg ttgatgaacc cgttccacgt gctgttgatc 240  
 gtgctcgtt atttgggtcac ggtctttgtg ggcattcaga tcatgaagaa ctttgagcgg 300  
 ttcgagggtca agacgttttc gctcctgcac aacttttgtc tggctctgat cagcgcctac 360  
 atgtgcggtg ggatcctgta cgaggcttat caggccaact atggactggt tgagaacgct 420  
 gctgatcata ccttcaaggg tcttccatag gccaatga tctggctctt ctacttctcc 480  
 aagatcatgg agtttgtcga caccatgatc atggctcctc agaagaacaa ccgccagatc 540  
 tccttcttgc acgtttacca ccacagctcc atcttcacca tctgggtggt ggtaaccttt 600  
 gttgcacca acggtgaagc ctacttctct gctgcgttga actcgttcat ccatgtgatc 660



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atgtacggct actacttctt gtcggccttg ggcttcaagc aggtgtcgtt catcaagttc 720
tacatcacgc gctcgcagat gacacagttc tgcattgatgt cgggtccagtc ttccctgggac 780
atgtacgcca tgaaggctct tggccgcccc ggatacccct tcttcatcac ggctctgctt 840
tggttctaca tgtggaccat gctcgggtctc ttctacaact ttacagaaa gaacgccaaag 900
ttggccaagc aggccaaggc cgacgctgcc aaggagaagg caaggaagtt gcagtaa 957

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<210> 23  
 <211> 30  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer R0936

<400> 23  
 gtcgggcaag gcggaaaagt acctcaagag 30

<210> 24  
 <211> 30  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer R0937

<400> 24  
 aaacctgtag acaatgtgga ggggcgtggg 30

<210> 25  
 <211> 42  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer R0972

<400> 25  
 atacttgaat tcatgggacg cggcggcgaa ggtcaggtga ac 42

<210> 26  
 <211> 39  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer R0949

<400> 26  
 cttatactcg agctaagcgg ccttggccgc cgcctggcc 39

<210> 27  
 <211> 41  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer R0950

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&lt;400&gt; 27

cttataactcg agtaaattggc tcgcgaggcg aagcgagtgg c

41

&lt;210&gt; 28

&lt;211&gt; 1320

&lt;212&gt; DNA

<213> *Thraustochytrium aureum*

&lt;400&gt; 28

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atgggacgcg gcggcgaagg tcaggtgaac agcgcgaggg tggcacaagg cgggtgcggga 60
acgcgaaaga cgatcctgat cgagggcgag gtctacgatg tcaccaactt taggcacccc 120
ggcgggtcga tcatcaagtt tctcacgacc gacggcaccg aggctgtgga cgcgacgaac 180
gcgttttcgcg agttttactg ccggtcgggc aaggcggaag agtacctcaa gagcctgccc 240
aagctcggcg cgccgagcaa gatgaagttt gacgcccaagg agcaggcccg gcgcgacgcg 300
atcacgcgag actacgtcaa gctgcgcgag gagatgggtg ccgagggcct cttcaagccc 360
gcgcccctcc acattgtcta caggttttgcg gagatcgcag ccctgttcgc ggcctcgttc 420
tacctgtttt cgatgcgcgg aaacgtgttc gccacgctcg cggccatcgc agtcgggggc 480
atcgcgaggg gccgctgcgg ctggctcatg cacgagtgcg gacacttctc gatgaccggg 540
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gacgtcgacc tcgacaccct gccgctcggt gcgttcaacg agaagatcgc cgccaagggtg 720
cgccccggct cgttccaggc caagtggctc tcggcgagg cgtacatttt tgcgcgggtg 780
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aacgactcgt ggttcatcac ctgggtggatg tcgtacctca actttcagat cgagcaccac 1140
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tttgccaacc tgcacgccgt gggccaaaac gcggggccagg cggcgggccaa ggccgcttag 1320

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&lt;210&gt; 29

&lt;211&gt; 439

&lt;212&gt; PRT

<213> *Thraustochytrium aureum*

&lt;400&gt; 29

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Met Gly Arg Gly Gly Glu Gly Gln Val Asn Ser Ala Gln Val Ala Gln
 1           5           10           15
Gly Gly Ala Gly Thr Arg Lys Thr Ile Leu Ile Glu Gly Glu Val Tyr
          20           25           30
Asp Val Thr Asn Phe Arg His Pro Gly Gly Ser Ile Ile Lys Phe Leu
          35           40           45
Thr Thr Asp Gly Thr Glu Ala Val Asp Ala Thr Asn Ala Phe Arg Glu
          50           55           60
Phe His Cys Arg Ser Gly Lys Ala Glu Lys Tyr Leu Lys Ser Leu Pro
65           70           75           80
Lys Leu Gly Ala Pro Ser Lys Met Lys Phe Asp Ala Lys Glu Gln Ala
          85           90           95
Arg Arg Asp Ala Ile Thr Arg Asp Tyr Val Lys Leu Arg Glu Glu Met
          100          105          110
Val Ala Glu Gly Leu Phe Lys Pro Ala Pro Leu His Ile Val Tyr Arg
          115          120          125
Phe Ala Glu Ile Ala Ala Leu Phe Ala Ala Ser Phe Tyr Leu Phe Ser

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130	135	140
Met Arg Gly Asn Val Phe Ala Thr Leu Ala Ala Ile Ala Val Gly Gly		
145	150	155
Ile Ala Gln Gly Arg Cys Gly Trp Leu Met His Glu Cys Gly His Phe		160
	165	170
Ser Met Thr Gly Tyr Ile Pro Leu Asp Val Arg Leu Gln Glu Leu Val		175
	180	185
Tyr Gly Val Gly Cys Ser Met Ser Ala Ser Trp Trp Arg Val Gln His		190
	195	200
Asn Lys His His Ala Thr Pro Gln Lys Leu Lys His Asp Val Asp Leu		205
	210	215
Asp Thr Leu Pro Leu Val Ala Phe Asn Glu Lys Ile Ala Ala Lys Val		220
225	230	235
Arg Pro Gly Ser Phe Gln Ala Lys Trp Leu Ser Ala Gln Ala Tyr Ile		240
	245	250
Phe Ala Pro Val Ser Cys Phe Leu Val Gly Leu Phe Trp Thr Leu Phe		255
	260	265
Leu His Pro Arg His Met Pro Arg Thr Ser His Phe Ala Glu Met Ala		270
	275	280
Ala Val Ala Val Arg Val Val Gly Trp Ala Ala Leu Met His Ser Phe		285
	290	295
Gly Tyr Ser Gly Ser Asp Ser Phe Gly Leu Tyr Met Ala Thr Phe Gly		300
305	310	315
Phe Gly Cys Thr Tyr Ile Phe Thr Asn Phe Ala Val Ser His Thr His		320
	325	330
Leu Asp Val Thr Glu Pro Asp Glu Phe Leu His Trp Val Glu Tyr Ala		335
	340	345
Ala Leu His Thr Thr Asn Val Ser Asn Asp Ser Trp Phe Ile Thr Trp		350
	355	360
Trp Met Ser Tyr Leu Asn Phe Gln Ile Glu His His Leu Phe Pro Ser		365
	370	375
Leu Pro Gln Leu Asn Ala Pro Arg Val Ala Pro Arg Val Arg Ala Leu		380
385	390	395
Phe Glu Lys His Gly Met Ala Tyr Asp Glu Arg Pro Tyr Leu Thr Ala		400
	405	410
Leu Gly Asp Thr Phe Ala Asn Leu His Ala Val Gly Gln Asn Ala Gly		415
	420	425
Gln Ala Ala Ala Lys Ala Ala		430
	435	

&lt;210&gt; 30

&lt;211&gt; 1338

&lt;212&gt; DNA

&lt;213&gt; Thraustochytrium aureum

&lt;400&gt; 30

gaattcacca	tgggtcgcgg	agcacaggga	gagccaaggc	aggccacaga	gctgaagagc	60
agcccaagtg	agcagcgtaa	ggtgttgctc	attgacgggc	agctgtacga	tgcaaccaac	120
ttcaggcatc	ctggtggctc	catcatcaaa	tattttgtgca	ccgatggcaa	ggaggtagtt	180
gatgcaaccg	aagcgtacaa	ggagttccac	tgcagatcct	cgaaggcggg	caagtacctc	240
aactccctgc	caaagatcga	cggcccaatc	aagtacaaat	acgacgcaaa	ggagcaggct	300
cgccatgaca	aactcacgag	ggagtatgta	gctctccgcg	aacagctcgt	caaggaggga	360
tactttgacc	ccagcccgtc	ccacattatc	tacagatgcg	ccgagttggc	agccatgttc	420
gctctctcgt	tctacctttt	ctccttcaag	ggtaacgtca	tggccactat	tgctgccatc	480
gtgattgggg	ggtgcgtgca	gggtcgttgt	gggtggctca	tgcatgaagc	tggccactac	540
agcatgaccg	gaaacatccc	tgttgacttg	cgccttcaag	agttttttgta	cggaattggg	600

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tgtggcatga gcggggcttg gtggagaagc cagcacaaca agcaccacgc cccccccaa      660
aagctcaagc atgacgttga tttggacact cttcctcttg tcgcctggaa cgagaaaatt      720
gcccgtcgcg tcaagccagg tagcttccag gcaaagtggc ttcattctcca gggatacatc      780
tttgccccag tctcctgcct tctcgttggt ctcttctgga ctttgtactt gcattctcgc      840
cacatgatcc gcaccaagcg caacttcgag atattttctg tcgctctgcg ctacgtatgc      900
tggttctcgc ttctttttgag catgggctac actgtcggag agtctctggg tctctatgtg      960
cttacttttg gacttggctg tacctacatc tttacgcatt ttgctgtaag ccacaccac      1020
ttgccagtgt ccgaggagga cgagtacctg cactgggtcg agtacgctgc gctgcacacc      1080
acgaacgttg ccatcgactc gtacgttgtc acctggctga tgagctacct caactttcag      1140
atcgagcacc acttgttccc ttgctgcccg cagttccgcc accctgcaat ctcttctcgc      1200
gtcaagaaac ttttcgagga caatggtctg gtatacgacg cccgctcata cgtccaggcg      1260
ctcaaggata ctttcggcaa cctacacgaa gtgggcgtca acgctggcca agctgccaag      1320
agcgagtaag atctcgag                                     1338

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&lt;210&gt; 31

&lt;211&gt; 439

&lt;212&gt; PRT

&lt;213&gt; Thraustochytrium aureum

&lt;400&gt; 31

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Met Gly Arg Gly Ala Gln Gly Glu Pro Arg Gln Ala Thr Glu Leu Lys
 1          5          10          15
Ser Ser Pro Ser Glu Gln Arg Lys Val Leu Leu Ile Asp Gly Gln Leu
          20          25          30
Tyr Asp Ala Thr Asn Phe Arg His Pro Gly Gly Ser Ile Ile Lys Tyr
          35          40          45
Leu Cys Thr Asp Gly Lys Glu Val Val Asp Ala Thr Glu Ala Tyr Lys
          50          55          60
Glu Phe His Cys Arg Ser Ser Lys Ala Val Lys Tyr Leu Asn Ser Leu
65          70          75          80
Pro Lys Ile Asp Gly Pro Ile Lys Tyr Lys Tyr Asp Ala Lys Glu Gln
          85          90          95
Ala Arg His Asp Lys Leu Thr Arg Glu Tyr Val Ala Leu Arg Glu Gln
          100          105          110
Leu Val Lys Glu Gly Tyr Phe Asp Pro Ser Pro Leu His Ile Ile Tyr
          115          120          125
Arg Cys Ala Glu Leu Ala Ala Met Phe Ala Leu Ser Phe Tyr Leu Phe
          130          135          140
Ser Phe Lys Gly Asn Val Met Ala Thr Ile Ala Ala Ile Val Ile Gly
145          150          155          160
Gly Cys Val Gln Gly Arg Cys Gly Trp Leu Met His Glu Ala Gly His
          165          170          175
Tyr Ser Met Thr Gly Asn Ile Pro Val Asp Leu Arg Leu Gln Glu Phe
          180          185          190
Leu Tyr Gly Ile Gly Cys Gly Met Ser Gly Ala Trp Trp Arg Ser Gln
          195          200          205
His Asn Lys His His Ala Thr Pro Gln Lys Leu Lys His Asp Val Asp
210          215          220
Leu Asp Thr Leu Pro Leu Val Ala Trp Asn Glu Lys Ile Ala Arg Arg
225          230          235          240
Val Lys Pro Gly Ser Phe Gln Ala Lys Trp Leu His Leu Gln Gly Tyr
          245          250          255
Ile Phe Ala Pro Val Ser Cys Leu Leu Val Gly Leu Phe Trp Thr Leu
          260          265          270
Tyr Leu His Pro Arg His Met Ile Arg Thr Lys Arg Asn Phe Glu Ile
          275          280          285
Phe Ser Val Ala Leu Arg Tyr Val Cys Trp Phe Ser Leu Leu Leu Ser

```

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290		295		300
Met Gly Tyr Thr Val	Gly Glu Ser Leu Gly Leu	Tyr Val Leu Thr Phe		
305		310		320
Gly Leu Gly Cys Thr	Tyr Ile Phe Thr His Phe	Ala Val Ser His Thr		
	325		330	335
His Leu Pro Val Ser	Glu Glu Asp Glu Tyr Leu	His Trp Val Glu Tyr		
	340		345	350
Ala Ala Leu His Thr	Thr Asn Val Ala Ile Asp	Ser Tyr Val Val Thr		
	355		360	365
Trp Leu Met Ser Tyr	Leu Asn Phe Gln Ile Glu	His His Leu Phe Pro		
	370		375	380
Cys Cys Pro Gln Phe	Arg His Pro Ala Ile Ser	Ser Arg Val Lys Lys		
385		390		400
Leu Phe Glu Asp Asn	Gly Leu Val Tyr Asp	Ala Arg Ser Tyr Val	Gln	
	405		410	415
Ala Leu Lys Asp Thr	Phe Gly Asn Leu His	Glu Val Gly Val Asn	Ala	
	420		425	430
Gly Gln Ala Ala Lys	Ser Glu			
	435			

<210> 32  
 <211> 1381  
 <212> DNA  
 <213> Thraustochytrium aureum

<400> 32

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agctccagaa	ggccaagtgg	gaggatgttg	ttcgcatcaa	tggagtcgaa	tacgacgtca	120
cggactatct	cagaaaacac	cctgggtggca	gcgtgatcaa	gtacgggctt	gccaacaccg	180
gcgctgatgc	cacgtccctc	tttgaagcgt	tccacatgcg	ctcaaagaag	gctcagatgg	240
tgctcaagtc	tctcccaaag	cgtgctccgg	tcctcgagat	ccagccaaac	cagcttccag	300
aggagcagac	caaggaggcg	gagatgctgc	gtgattttta	aaaatttgag	gatgagattc	360
gccgggatgg	attgatggaa	ccttcccttct	ggcatcgcg	ttacagatta	tcagagcttg	420
taggtatgtt	cacgctcggc	ctctacctct	tctcgtttaa	cactcctctg	tctattgctg	480
ctggtgtcct	cgtccacggt	ctcttttggtg	cattctgtgg	atggtgccag	catgaggcag	540
gccacggctc	cttttttttac	agcctttggt	ggggcaagcg	tgtacaggcc	atggtgatcg	600
ggtttggtct	aggaacatcc	ggcgacatgt	ggaacatgat	gcacaacaag	catcatgctg	660
ccacccaaaa	ggttcatcac	gaccttgaca	ttgacacaac	tcctttttgta	gcttttcttca	720
acactgcatt	tgagaaaaaac	agatggaagg	gctttttccaa	ggcttggggtc	cgcttttcagg	780
cttttcacgtt	cattcctgtc	accagcgcca	tgatcgatcat	gctgttctgg	ctgttttttct	840
tccaccctcg	ccgcgtcggt	caaaagaaga	actttgagga	gggttttttgg	atgctgtcga	900
gccacattgt	gcgcacctat	ctcttccacc	ttgtgaccgg	ctgggagagc	ctcgctgcat	960
gctaccttgt	tgggtatttg	gcgtgcatgt	gggtgtccgg	tatgtatttg	tttggccact	1020
tttcgctctc	ccacactcat	atggacattg	tggaggcgga	cgtgcataag	aactgggtca	1080
ggtacgctgt	tgaccacact	gttgacatca	gcccatacaa	cccgtcgtg	tgctgggtca	1140
tgggttacct	caacatgcag	accatccacc	acttgtggcc	tgccatgcc	cagtaccacc	1200
aggtcgaggt	ctcacgccgc	tttgccatct	tcgccaaaaa	acacggcctc	aactaccgcg	1260
tcgtctctta	ctttgaggct	tggcgccctga	tgctccaaaa	tcttgctgac	gtcggttccc	1320
actacatga	gaacggtgtc	aagcgcgccc	caaagaaagc	caaggcgag	tagaaagcta	1380
t						1381

<210> 33  
 <211> 456  
 <212> PRT  
 <213> Thraustochytrium aureum

<400> 33

Met 1	Gly	Arg	Gly	Gly 5	Glu	Lys	Ser	Glu	Val 10	Asp	Gln	Val	Gln	Pro 15	Gln
Lys	Thr	Glu	Gln 20	Leu	Gln	Lys	Ala	Lys 25	Trp	Glu	Asp	Val	Val 30	Arg	Ile
Asn	Gly	Val 35	Glu	Tyr	Asp	Val	Thr 40	Asp	Tyr	Leu	Arg	Lys 45	His	Pro	Gly
Gly	Ser 50	Val	Ile	Lys	Tyr	Gly 55	Leu	Ala	Asn	Thr 60	Gly	Ala	Asp	Ala	Thr
Ser 65	Leu	Phe	Glu	Ala	Phe 70	His	Met	Arg	Ser	Lys 75	Lys	Ala	Gln	Met	Val 80
Leu	Lys	Ser	Leu	Pro 85	Lys	Arg	Ala	Pro	Val 90	Leu	Glu	Ile	Gln	Pro 95	Asn
Gln	Leu	Pro	Glu 100	Glu	Gln	Thr	Lys	Glu 105	Ala	Glu	Met	Leu	Arg 110	Asp	Phe
Lys	Lys	Phe 115	Glu	Asp	Glu	Ile	Arg 120	Arg	Asp	Gly	Leu	Met 125	Glu	Pro	Ser
Phe	Trp 130	His	Arg	Ala	Tyr	Arg 135	Leu	Ser	Glu	Leu 140	Val	Gly	Met	Phe	Thr
Leu 145	Gly	Leu	Tyr	Leu	Phe 150	Ser	Leu	Asn	Thr	Pro 155	Leu	Ser	Ile	Ala	Ala 160
Gly	Val	Leu	Val 165	His	Gly	Leu	Phe	Gly 170	Ala	Phe	Cys	Gly	Trp	Cys 175	Gln
His	Glu	Ala	Gly 180	His	Gly	Ser	Phe	Phe 185	Tyr	Ser	Leu	Trp	Trp 190	Gly	Lys
Arg	Val	Gln 195	Ala	Met	Leu	Ile	Gly 200	Phe	Gly	Leu	Gly	Thr 205	Ser	Gly	Asp
Met	Trp 210	Asn	Met	Met	His	Asn 215	Lys	His	His	Ala 220	Ala	Thr	Gln	Lys	Val
His 225	His	Asp	Leu	Asp	Ile 230	Asp	Thr	Thr	Pro	Phe 235	Val	Ala	Phe	Phe	Asn 240
Thr	Ala	Phe	Glu 245	Lys	Asn	Arg	Trp	Lys	Gly 250	Phe	Ser	Lys	Ala	Trp 255	Val
Arg	Phe	Gln 260	Ala	Phe	Thr	Phe	Ile	Pro 265	Val	Thr	Ser	Gly 270	Met	Ile	Val
Met	Leu	Phe 275	Trp	Leu	Phe	Phe	Leu	His 280	Pro	Arg	Arg	Val 285	Val	Gln	Lys
Lys	Asn 290	Phe	Glu	Glu	Gly	Phe 295	Trp	Met	Leu	Ser	Ser 300	His	Ile	Val	Arg
Thr 305	Tyr	Leu	Phe	His	Leu 310	Val	Thr	Gly	Trp	Glu 315	Ser	Leu	Ala	Ala	Cys 320
Tyr	Leu	Val	Gly 325	Tyr	Trp	Ala	Cys	Met	Trp 330	Val	Ser	Gly	Met	Tyr 335	Leu
Phe	Gly	His 340	Phe	Ser	Leu	Ser	His	Thr 345	His	Met	Asp	Ile 350	Val	Glu	Ala
Asp	Val	His 355	Lys	Asn	Trp	Val	Arg 360	Tyr	Ala	Val	Asp	His 365	Thr	Val	Asp
Ile	Ser 370	Pro	Ser	Asn	Pro	Leu 375	Val	Cys	Trp	Val	Met 380	Gly	Tyr	Leu	Asn
Met 385	Gln	Thr	Ile	His	His 390	Leu	Trp	Pro	Ala	Met 395	Pro	Gln	Tyr	His	Gln 400
Val	Glu	Val	Ser 405	Arg	Arg	Phe	Ala	Ile	Phe 410	Ala	Lys	Lys	His	Gly 415	Leu
Asn	Tyr	Arg 420	Val	Val	Ser	Tyr	Phe	Glu 425	Ala	Trp	Arg	Leu 430	Met	Leu	Gln
Asn	Leu	Ala 435	Asp	Val	Gly	Ser	His 440	Tyr	His	Glu	Asn	Gly 445	Val	Lys	Arg
Ala	Pro	Lys	Lys	Ala	Lys	Ala	Gln								



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450

455

<210> 34  
 <211> 1329  
 <212> DNA  
 <213> *Isochrysis galbana*

<400> 34  
 atggtggcag gcaaatacagg cgctgcggcg cacgtgactc acagctcgac attgccccgt 60  
 gagtaccatg gcgcgaccaa cgactcgcgc tctgaggcgg ccgacgtcac cgtctctagc 120  
 atcgatgctg aaaaggagat gatcatcaac ggccgcgtgt atgacgtgtc gtcattttgtg 180  
 aagcggcacc caggtggctc ggtgatcaag ttccagctgg gcgccgacgc gagecgacgcg 240  
 tacaacaact ttcacgtccg ctccaagaag gcggacaaga tgctgtattc gctcccgtcc 300  
 cggccggccg aggccggcta cgcccaggac gacatctccc gcgactttga gaagctgcgc 360  
 ctcgagctga aggaggaggg ctacttcgag cccaacctgg tgcacgtgag ctacaggtgt 420  
 gtggagggtc ttgccatgta ctgggctggc gtccagctca tctgggtccg gtactgggtc 480  
 ctcggcgcga tcgtggccgg cattgcgcag ggccgctgcg gctggctcca gcatgagggt 540  
 gggcactact cgctcaccgg caacatcaag atcgaccggc atctgcagat ggccatctat 600  
 gggcttggtt gcggcatgtc gggctgctac tggcgcaacc agcacaacaa gcaccacgcc 660  
 acgccgcaga agctcgggac cgaccccgac ctgcagacga tgccgctggg ggccttccac 720  
 aagatcgctg gcgccaaggc gcgaggcaag ggcaaggcgt ggctggcgtg gcaggcgccg 780  
 ctcttctttg gcgggatcat ctgctcgctc gtctctttcg gctggcagtt cgtgctccac 840  
 cccaaccacg cgctgcgcgt gcacaatcac ctggagctcg cgtacatggg cctgcggtac 900  
 gtgctgtggc acctggcctt tggccacctc gggctgctga gctcgctccg cctgtacgcc 960  
 ttttacgtgg ccgtggggcg cacctacatc ttaccaact tcgccgtctc gcacaccac 1020  
 aaggacgtcg tcccggccac caagcacatc tcgtggggc tctactcggc caaccacacg 1080  
 accaactgct ccgactcgcc ctttgtcaac tgggtggatg cctacctcaa cttccagatc 1140  
 gagcaccacc tcttcccgtc gatgccgcag tacaaccacc ccaagatcgc cccgcgggtg 1200  
 cgcgcgctct tcgagaagca cggggctcag tatgacgtcc ggccatacct ggagtgtttt 1260  
 cgggtcacgt acgtcaacct gctcgccgta ggcaaccgg agcactccta ccacgagcac 1320  
 acgcactag 1329

<210> 35  
 <211> 442  
 <212> PRT  
 <213> *Isochrysis galbana*

<400> 35  
 Met Val Ala Gly Lys Ser Gly Ala Ala Ala His Val Thr His Ser Ser  
 1 5 10 15  
 Thr Leu Pro Arg Glu Tyr His Gly Ala Thr Asn Asp Ser Arg Ser Glu  
 20 25 30  
 Ala Ala Asp Val Thr Val Ser Ser Ile Asp Ala Glu Lys Glu Met Ile  
 35 40 45  
 Ile Asn Gly Arg Val Tyr Asp Val Ser Ser Phe Val Lys Arg His Pro  
 50 55 60  
 Gly Gly Ser Val Ile Lys Phe Gln Leu Gly Ala Asp Ala Ser Asp Ala  
 65 70 75 80  
 Tyr Asn Asn Phe His Val Arg Ser Lys Lys Ala Asp Lys Met Leu Tyr  
 85 90 95  
 Ser Leu Pro Ser Arg Pro Ala Glu Ala Gly Tyr Ala Gln Asp Asp Ile  
 100 105 110  
 Ser Arg Asp Phe Glu Lys Leu Arg Leu Glu Leu Lys Glu Glu Gly Tyr  
 115 120 125  
 Phe Glu Pro Asn Leu Val His Val Ser Tyr Arg Cys Val Glu Val Leu  
 130 135 140  
 Ala Met Tyr Trp Ala Gly Val Gln Leu Ile Trp Ser Gly Tyr Trp Phe



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145					150					155				160	
Leu	Gly	Ala	Ile	Val	Ala	Gly	Ile	Ala	Gln	Gly	Arg	Cys	Gly	Trp	Leu
				165					170					175	
Gln	His	Glu	Gly	Gly	His	Tyr	Ser	Leu	Thr	Gly	Asn	Ile	Lys	Ile	Asp
			180					185					190		
Arg	His	Leu	Gln	Met	Ala	Ile	Tyr	Gly	Leu	Gly	Cys	Gly	Met	Ser	Gly
		195					200					205			
Cys	Tyr	Trp	Arg	Asn	Gln	His	Asn	Lys	His	His	Ala	Thr	Pro	Gln	Lys
	210					215					220				
Leu	Gly	Thr	Asp	Pro	Asp	Leu	Gln	Thr	Met	Pro	Leu	Val	Ala	Phe	His
225					230					235					240
Lys	Ile	Val	Gly	Ala	Lys	Ala	Arg	Gly	Lys	Gly	Lys	Ala	Trp	Leu	Ala
			245					250						255	
Trp	Gln	Ala	Pro	Leu	Phe	Phe	Gly	Gly	Ile	Ile	Cys	Ser	Leu	Val	Ser
			260					265					270		
Phe	Gly	Trp	Gln	Phe	Val	Leu	His	Pro	Asn	His	Ala	Leu	Arg	Val	His
	275						280					285			
Asn	His	Leu	Glu	Leu	Ala	Tyr	Met	Gly	Leu	Arg	Tyr	Val	Leu	Trp	His
	290					295					300				
Leu	Ala	Phe	Gly	His	Leu	Gly	Leu	Leu	Ser	Ser	Leu	Arg	Leu	Tyr	Ala
305					310					315					320
Phe	Tyr	Val	Ala	Val	Gly	Gly	Thr	Tyr	Ile	Phe	Thr	Asn	Phe	Ala	Val
			325					330					335		
Ser	His	Thr	His	Lys	Asp	Val	Val	Pro	Pro	Thr	Lys	His	Ile	Ser	Trp
			340					345					350		
Ala	Leu	Tyr	Ser	Ala	Asn	His	Thr	Thr	Asn	Cys	Ser	Asp	Ser	Pro	Phe
	355						360					365			
Val	Asn	Trp	Trp	Met	Ala	Tyr	Leu	Asn	Phe	Gln	Ile	Glu	His	His	Leu
	370					375					380				
Phe	Pro	Ser	Met	Pro	Gln	Tyr	Asn	His	Pro	Lys	Ile	Ala	Pro	Arg	Val
385					390					395					400
Arg	Ala	Leu	Phe	Glu	Lys	His	Gly	Val	Glu	Tyr	Asp	Val	Arg	Pro	Tyr
			405					410					415		
Leu	Glu	Cys	Phe	Arg	Val	Thr	Tyr	Val	Asn	Leu	Leu	Ala	Val	Gly	Asn
			420				425						430		
Pro	Glu	His	Ser	Tyr	His	Glu	His	Thr	His						
	435						440								

&lt;210&gt; 36

&lt;211&gt; 36

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer RO838

&lt;221&gt; misc\_feature

&lt;222&gt; (7)...(7)

&lt;223&gt; v = a or g or c at position 7

&lt;221&gt; misc\_feature

&lt;222&gt; (10)...(10)

&lt;223&gt; r = g or a at position 10

&lt;221&gt; misc\_feature

&lt;222&gt; (13)...(13)

&lt;223&gt; s = g or c at position 13

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<221> misc\_feature  
 <222> (16)...(16)  
 <223> r = g or a at position 16

<221> misc\_feature  
 <222> (19)...(19)  
 <223> r = g or a at position 19

<221> misc\_feature  
 <222> (22)...(22)  
 <223> y = t/u or c at position 22

<221> misc\_feature  
 <222> (25)...(25)  
 <223> r = g or a at position 25

<221> misc\_feature  
 <222> (31)...(31)  
 <223> r = g or a at position 31

<400> 36  
 catggtvggr aasagrtgrt gytcratctg rtagtt 36

<210> 37  
 <211> 25  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer RO1065

<400> 37  
 cgacaagagg aagagtgtcc aaatc 25

<210> 38  
 <211> 30  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer RO1064

<400> 38  
 cgcottcaag agttttttgta cggaattggg 30

<210> 39  
 <211> 30  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer RO1097

<400> 39  
 cttgtaccat gggtcgcgga gcacagggag 30

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<210> 40  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer RO1098

<400> 40  
tgaagcttac tcgctcttgg cagcttggcc 30

<210> 41  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer RO1107

<400> 41  
tttaaccatg ggccgcggcg gcgagaaaag 30

<210> 42  
<211> 36  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer RO1108

<400> 42  
gggaagaagc tttctactgc gccttggctt tctttg 36

<210> 43  
<211> 26  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer RO1235

<400> 43  
cgaagttggt gaagatgtag gtgccg 26

<210> 44  
<211> 27  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer RO1232

<400> 44  
gagcgacgcg tacaacaact ttcacgt 27

<210> 45  
<211> 23

22/24

<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 45  
cgactggagc acgaggacac tga

<210> 46  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> GeneRacer 3 Prime Primer

<400> 46  
gctgtcaacg atacgctacg taacg

<210> 47  
<211> 26  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Nested Primer R01234

<400> 47  
agctccaggt gattgtgcac gcgcag

<210> 48  
<211> 27  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer R01233

<400> 48  
gactttgaga agctgcgcct cgagctg

<210> 49  
<211> 26  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Nested 5 Prime Primer

<400> 49  
ggacactgac atggactgaa ggagta

<210> 50  
<211> 23  
<212> DNA  
<213> Artificial Sequence

23

25

26

27

26

23/24

<220>  
 <223> Nested 3 Prime Primer

<400> 50  
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<210> 51  
 <211> 35  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer RO1309

<400> 51  
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<210> 52  
 <211> 35  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer RO1310

<400> 52  
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<210> 53  
 <211> 14  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Consensus Peptide Sequence

<400> 53  
 Val Tyr Asp Val Thr Glu Trp Val Lys Arg His Pro Gly Gly  
 1 5 10

<210> 54  
 <211> 15  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Consensus Peptide Sequence

<400> 54  
 Gly Ala Ser Ala Asn Trp Trp Lys His Gln His Asn Val His His  
 1 5 10 15

<210> 55  
 <211> 12  
 <212> PRT  
 <213> Artificial Sequence

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&lt;220&gt;

&lt;223&gt; Consensus Peptide Sequence

&lt;400&gt; 55

Asn	Tyr	Gln	Ile	Glu	His	His	Leu	Phe	Pro	Thr	Met
1				5					10		